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ABSTRACTS OF THE 190TH AMERICAN CHEMICAL SOCIETY NATIONAL MEETING, vol. 190,1985, page 23, no. 47; R.R. BOTT et al.: "Protein engineering of subtilisin"

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Description

The recent development of various in vitro techniques to manipulate the DNA sequences encoding naturally-occuring polypeptides as well as recent developments in the chemical synthesis of relatively short sequences of single and double stranded DNA has resulted in the speculation that such techniques can be used to modify enzymes to improve some functional property in a predictable way. Ulmer, K.M. (1983) Science 219, 666-671. The only working example disclosed therein is the substitution of a single amino acid within the active site of tyrosyl-tRNA synthetase (Cys35→Ser) which lead to a reduction in enzymatic activity. See Winter, G., et al. (1982) Nature 299, 756-758; and Wilkinson, A.J., et al. (1983) Biochemistry 22, 3581-3586 (Cys35→Gly mutation also resulted in decreased activity).

When the same t-RNA synthetase was modified by substituting a different amino acid residue within the active site with two different amino acids, one of the mutants (Thr51→Ala) reportedly demonstrated a predicted moderate increase in kcat/Km whereas a second mutant (Thr51→Pro) demonstrated a massive increase in kcat/Km which could not be explained with certainty. Wilkinson, A.H., et al. (1984) Nature 307, 187-188.

Another reported example of a single substitution of an amino acid residue is the substitution of cysteine for isoleucine at the third residue of T4 lysozyme. Perry, L.J., et al. (1984) Science 226, 555-557. The resultant mutant lysozyme was mildly oxidized to form a disulfide bond between the new cysteine residue at position 3 and the native cysteine at position 97. This crosslinked mutant was initially described by the author as being enzymatically identical to, but more thermally stable than, the wild type enzyme. However, in a "Note Added in Proof", the author indicated that the enhanced stability observed was probably due to a chemical modification of cysteine at residue 54 since the mutant lysozyme with a free thiol at Cys54 has a thermal stability identical to the wild type lysozyme.

Similarly, a modified dihydrofolate reductase from E.coli has been reported to be modified by similar methods to introduce a cysteine which could be cross linked with a naturally-occurring cysteine in the reductase. Villafranca, D.E., et al. (1983) Science 222, 782-788. The author indicates that this mutant is fully reactive in the reduced state but has significantly diminished activity in the oxidized state. In addition, two other substitutions of specific amino acid residues are reported which resulted in mutants which had diminished or no activity.

EPO Publication No. 0130756 discloses the substitution of specific residues within B. amyloliquefaciens subtilisin with specific amino acids. Thus, Met222 has been substituted with all 19 other amino acids, Gly166 with 9 different amino acids and Gly169 with Ala and Ser.

As set forth below, several laboratories have also reported the use of site directed mutagensis to produce the mutation of more than one amino acid residue within a polypeptide.

The amino-terminal region of the signal peptide of the prolipoprotein of the E. coli outer membrane was stated to be altered by the substitution or deletion of residues 2 and 3 to produce a charge change in that region of the polypeptide. Inoyye, S., et al. (1982) Proc. Nat. Acad. Sci. USA 79, 3438-3441. The same laboratory also reported the substitution and deletion of amino acid redisues 9 and 14 to determine the effects of such substitution on the hydrophobic region of the same signal sequence. Inouye, S., et al. (1984) J. Biol. Chem. 259, 3729-3733.

Double mutants in the active site of tyrosyl-t-RNA synthetase have also been reported. Carter, P.J., et al. (1984) Cell 38, 835-840. In this report, the improved affinity of the previously described Thr51→Pro mutant for ATP was probed by producing a second mutation in the active site of the enzyme. One of the double mutants, Gly35/Pro51, reportedly demonstrated an unexpected result in that it bound ATP in the transition state better than was expected from the two single mutants. Moreover, the author warns, at least for one double mutant, that it is not readily predictable how one substitution alters the effect caused by the other substitution and that care must be taken in interpreting such substitutions.

A mutant is disclosed in U.S. Patent No. 4,532,207, wherein a polyarginine tail was attached to the C-terminal residue of β-urogastrone by modifying the DNA sequence encoding the polypeptide. As disclosed, the polyarginine tail changed the electrophoretic mobility of the urogastrone-polyaginine hybrid permiting selective purification. The polyarginine was subsequently removed, according to the patentee, by a polyarginine specific exopeptidase to produce the purified urogastrone. Properly construed, this reference discloses hybrid polypeptides which do not constitute mutant polypeptides containing the substitution, insertion or deletion of one or more amino acids of a naturally occurring polypeptide.

Single and double mutants of rat pancreatic trypsin have also been reported. Craik, C.S., et al. (1985) Science 228, 291-297. As reported, glycine residues at positions 216 and 226 were replaced with alanine residues to produce three trypsin mutants (two single mutants and one double mutant). In the case of the single mutants, the authors stated expectation was to observe a differential effect on Km. They instead

reported a change in specificity (kcat/Km) which was primarily the result of a decrease in kcat. In contrast, the double mutant reportedly demonstrated a differential increase in Km for lysyl and arginyl substrates as compared to wild type trypsin but had virtually no catalytic activity.

The references discussed above are provided solely for their disclosure prior to the filing date of the instant case, and nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or priority based on earlier filed applications.

Based on the above references, however, it is apparent that the modification of the amino acid sequence of wild type enzymes often results in the decrease or destruction of biological activity.

Accordingly, it is an object herein to provide carbonyl hydrolase mutants which have at least one property which is different from the same property of the carbonyl hydrolase precursor from which the amino acid of said mutant is derived.

It is a further object to provide mutant DNA sequences encoding such carbonyl hydrolase mutants as well as expression vectors containing such mutant DNA sequences.

Still further, another object of the present invention is to provide host cells transformed with such vectors as well as host cells which are capable of expressing such mutants either intracellularly or extracellularly.

Summary of the Invention

The invention includes carbonyl hydrolase mutants, preferably having at least one property which is substantially different from the same property of the precursor non-human carbonyl hydrolase from which the amino acid sequence of the mutant is derived. These properties include oxidative stability, substrate, specificity catalytic activity, thermal stability, alkaline stability, pH activity profile and resistance to proteolytic degradation. The precursor carbonyl hydrolase may be naturally occurring carbonyl hydrolases or recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is derived by the substitution, deletion or insertion of one or more amino acids of the precursor carbonyl hydrolase amino acid sequence.

The invention also includes mutant DNA sequences encoding such carbonyl hydrolase mutants. Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing said carbonyl hydrolase mutants.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the coding strand, correlated with the amino acid sequence of B. amyloliquefaciens subtilisin gene. Promoter (p) ribosome binding site (rbs) and termination (term) regions of the DNA sequence as well as sequences encoding the presequence (PRE) putative prosequence (PRO) and mature form (MAT) of the hydrolase are also shown.

Figure 2 is a schematic diagram showing the substrate binding cleft of subtilisin together with substrate.

Figure 3 is a stereo view of the S-1 binding subsite of B. amyloliquefaciens subtilisin showing a lysine P-1 substrate bound in the site in two different ways. Figure 3A shows Lysine P-1 substrate bound to form a salt bridge with a Glu at position 156. Figure 3B shows Lysine P-1 substrate bound to form a salt bridge with Glu at position 166.

Figure 4 is a schematic diagram of the active site of subtilisin Asp32, His64 and Ser221.

Figures 5A and 5B depict the amino acid sequence of subtilisin obtained from various sources. The residues directly beneath each residue of B. amyloliquefaciens subtilisin are equivalent residues which (1) can be mutated in a similar manner to that described for B. amyloliquefaciens subtilisin, or (2) can be used as a replacement amino acid residue in B. amyloliquefaciens subtilisin. Figure 5C depicts conserved residues of B. amyloliquefaciens subtilisin when compared to other subtilisin sequences.

Figures 6A and 6B depict the inactivation of the mutants Met222L and Met222Q when exposed to various organic oxidants.

Figure 7 depicts the ultraviolet spectrum of Met222F subtilisin and the difference spectrum generated after inactivation by diperdodecanoic acid (DPDA).

Figure 8 shows the pattern of cyanogen bromide digests of untreated and DPDA oxidized subtilisin Met222F on high resolution SDS-pyridine peptide gels.

Figure 9 depicts a map of the cyanogen bromide fragments of Fig. 8 and their alignment with the sequence of subtilisin Met222F.

Figure 10 depicts the construction of mutations between codons 45 and 50 of B. amyloliquefaciens subtilisin.

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Figure 11 depicts the construction of mutations between codons 122 and 127 of B. amyloliquefaciens subtilisin.

Figure 12 depicts the effect of DPDA on the activity of subtilisin mutants at positions 50 and 124 in subtilisin Met222F.

Figure 13 depicts the construction of mutations at codon 166 of B. amyloliquefaciens subtilisin.

Figure 14 depicts the effect of hydrophobicity of the P-1 substrate side-chain on the kinetic parameters of wild-type B. amyloliquefaciens subtilisin.

Figure 15 depicts the effect of position 166 side-chain substitutions on P-I substrate specificity. Figure 15A shows position 166 mutant subtilisins containing non-branched alkyl and aromatic side-chain substitutions arranged in order of increasing molecular volume. Figure 15B shows a series of mutant enzymes progressing through β- and γ-branched aliphatic side chain substitutions of increasing molecular volume.

Figure 16 depicts the effect of position 166 side-chain volumn on log kcat/Km for various P-1 substrates.

Figure 17 shows the substrate specificity differences between Ile166 and wild-type (Gly166) B. amyloliquefaciens subtilisin against a series of alphatic and aromatic substrates. Each bar represents the difference in log kcat/Km for Ile166 minus wild-type (Gly166) subtilisin.

Figure 18 depicts the construction of mutations at codon 169 of B. amyloliquefaciens subtilisin.

Figure 19 depicts the construction of mutations at codon 104 of B. amyloliquefaciens subtilisin.

Figure 20 depicts the construction of mutations at codon 152 B. amyloliquefaciens subtilisin.

Figure 21 depicts the construction of single mutations at codon 156 and double mutations at codons 156 and 166 of B. amyloliquefaciens subtilisin.

Figure 22 depicts the construction of mutations at codon 217 for B. amyloliquefaciens subtilisin.

Figure 23 depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 23A depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 24 depicts the kcat/Km versus pH profile for mutations at codon 222 in B. amyloliquefaciens subtilisin.

Figure 25 depicts the constructing mutants at codons 94, 95 and 96.

Figures 26 and 27 depict substrate specificity of various wild type and mutant subtilisins for different substrates.

Figures 28 A, B, C and D depict the effect of charge in the P-1 binding sites due to substitutions at codon 156 and 166.

Figures 29 A and B are a stereoview of the P-1 binding site of subtilisin BPN' showing a lysine P-1 substrate bound in the site in two ways. In 29A, Lysine P-1 substrate is built to form a salt bridge with a Glu at codon 156. In 29B, Lysine P-1 substrate is built to form a salt bridge with Glu at codon 166.

Figure 30 demonstrates residual enzyme activity versus temperature curves for purified wild-type (Panel A), C22/C87 (Panel B) and C24/C87 (Panel C).

Figure 31 depicts the strategy for producing point mutations in the subtilisin coding sequence by misincorporation of °-thioldeoxynucleotide triphosphates.

Figure 32 depicts the autolytic stability of purified wild type and mutant subtilisins 170E, 107V, 213R and 107V/213R at alkaline pH.

Figure 33 depicts the autolytic stability of purified wild type and mutant subtilisins V50, F50 and F50/V107/R213 at alkaline pH.

Figure 34 depicts the strategy for constructing plasmids containing random cassette mutagenesis over residues 197 through 228.

Figure 35 depicts the oligodeoxynucleotides used for random cassette mutagenesis over residues 197 through 228.

Figure 36 depicts the construction of mutants at codon 204.

Figure 37 depicts the oligodeoxynucleotides used for synthesizing mutants at codon 204.

Detailed Description

The inventors have discovered that various single and multiple in vitro mutations involving the substitution, deletion or insertion of one or more amino acids within a non-human carbonyl hydrolase amino acid sequence can confer advantageous properties to such mutants when compared to the non-mutated carbonyl hydrolase.

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Specifically, B. amyloliquefaciens subtilisin, an alkaline bacterial protease, has been mutated by modifying the DNA encoding the subtilisin to encode the substitution of one or more amino acids at various amino acid residues within the mature form of the subtilisin molecule. These in vitro mutant subtilisins have at least one property which is different when compared to the same property of the precursor subtilisin. These modified properties fall into several categories including: oxidative stability, substrate specificity, thermal stability, alkaline stability, catalytic activity, pH activity profile, resistance to proteolytic degradation, Km, kcat and Km/kcat ratio.

Carbonyl hydrolases are enzymes which hydrolyze compounds containing

bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally occurring carbonyl hydrolases principally include hydrolases, e.g. lipases and peptide hydrolases, e.g. subtilisins or metalloproteases. Peptide hydrolases include α-aminoacylpeptide hydrolase, peptidylamino-acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exoproteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein and in EPO Publication No. 0130756 published January 9, 1985.

Subtilisins are bacterial carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally occurring subtilisin or a recombinant subtilisin. A series of naturally occurring subtilisins is known to be produced and often secreted by various bacterial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus is aspartate-histidineserine. In the chymotrypsin related proteases the relative order, however is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a mutant DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed herein and in EPO Publication No. 0130756. For example, the subtilisin multiple mutant herein containing the substitution of methionine at amino acid residues 50, 124 and 222 with phenylalanine, isoleucine and glutamine, respectively, can be considered to be derived from the recombinant subtilisin containing the substitution of glutamine at residue 222 (Q222) disclosed in EPO Publication No. 0130756. The multiple mutant thus is produced by the substitution of phenylalanine for methionine at residue 50 and isoleucine for methionine at residue 124 in the Q222 recombinant subtilisin.

"Carbonyl hydrolases" and their genes may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as E. coli or pseudomonas and gram positive bacteria such as micrococcus or bacillus. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as S. cerevisiae, fungi such as Aspergillus sp., and non-human mammalian sources such as, for example, Bovine sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and non-human eucaryotic sources.

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A "carbonyl hydrolase mutant" has an amino acid sequence which is derived from the amino acid sequence of a non-human "precursor carbonyl hydrolase". The precursor carbonyl hydrolases include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase rathern than manipulation of the precursor carbonyl hydrolase per se. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in EPO Publication No. 0130756.

Specific residues of B. amyloliquefaciens subtilisin are identified for substitution, insertion or deletion. These amino acid position numbers refer to those assigned to the B. amyloliquefaciens subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues which are "equivalent" to the particular identified residues in B. amyloliquefaciens subtilisin.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of B. amyloliquefaciens subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analagous to a specific residue or portion of that residue in B. amyloliquefaciens subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly comparted to the B. amyloliquefaciens subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which sequence is known (Figure 5C). After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of B. amyloliquefaciens subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Figure 5A the amino acid sequence of subtilisin from B. amyloliquefaciens B. subtilisin var. I168 and B. lichenformis (carlsbergensis) are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These residues are identified in Fig. 5C.

These conserved residues thus may be used to define the corresponding equivalent amino acid residues of B. amyloliquefaciens subtilisin in other carbonyl hydrolases such as thermitase derived from Thermoactinomyces. These two particular sequences are aligned in Fig. 5B to produce the maximum homology of conserved residues. As can be seen there are a number of insertions and deletions in the thermitase sequence as compared to B. amyloliquefaciens subtilisin. Thus, in thermitase the equivalent amino acid of Tyr217 in B. amyloliquefaciens subtilisin is the particular lysine shown beneath Tyr217.

In Fig. 5A, the equivalent amino acid at position 217 in B. amyloliquefaciens subtilisin is Tyr. Likewise, in B. subtilis subtilisin position 217 is also occupied by Tyr but in B. licheniformis position 217 is occupied by Leu.

Thus, these particular residues in thermitase, and subtilisin from B. subtilisin and B. licheniformis may be substituted by a different amino acid to produce a mutant carbonyl hydrolase since they are equivalent in primary structure to Tyr217 in B. amyloliquefaciens subtilisin. Equivalent amino acids of course are not limited to those for Tyr217 but extend to any residue which is equivalent to a residue in B. amyloliquefaciens whether such residues are conserved or not.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and B. amyloliquefaciens subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the B. amyloliquefaciens subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

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$$\text{R factor} = \frac{\sum_h |Fo(h)| - |Fc(h)|}{\sum_h |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of B. amyloliquefaciens subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the B. amyloliquefaciens subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of B. amyloliquefaciens subtilisin. The three dimensional structures would be aligned as outlined above.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a mutant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally occurring sequence. The carbonyl hydrolase mutants of the present invention include the mature forms of carbonyl hydrolase mutants as well as the pro- and prepro-forms of such hydrolase mutants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase mutants.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in EPO Publication No. 0130756 to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the Bacillus strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in EPO Publicatin No. 0130756 and further described by Yang, M.Y., et al. (1984) J. Bacteriol. 160, 15-21. Other host cells for expressing subtilisin include Bacillus subtilis I168 (EPO Publication No. 0130756).

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the carbonyl hydrolase mutants or expressing the desired carbonyl hydrolase mutant. In the case of vectors which encode the pre or prepro form of the carbonyl hydrolase mutant, such mutants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods described herein in EPO publication No. 0130756.

Once the carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in EPO

Publication No. 0130756 and the production of carbonyl hydrolase mutants described herein.

The carbonyl hydrolase mutants of the present invention may be generated by site specific mutagenesis (Smith, M. (1985) Ann. Rev. Genet. 423; Zoeller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500), cassette mutagenesis (EPO Publication No. 0130756) or random mutagenesis (Shortle, D., et al. (1985) Genetics, 110, 539; Shortle, D., et al. (1986) Proteins: Structure, Function and Genetics, 1, 81; Shortle, D. (1986) J. Cell. Biochem, 30, 281; Alber, T., et al. (1985) Proc. Natl. Acad. of Sci., 82, 747; Matsumura, M., et al. (1985) J. Biochem., 260, 15298; Liao, H., et al. (1986) Proc. Natl. Acad. of Sci., 83 576) of the cloned precursor carbonyl hydrolase. Cassette mutagenesis and the random mutagenesis method disclosed herein are preferred.

The mutant carbonyl hydrolases expressed upon transformation of suitable hosts are screened for enzymes exhibiting one or more properties which are substantially different from the properties of the precursor carbonyl hydrolases, e.g., changes in substrate specificity, oxidative stability, thermal stability, alkaline stability, resistance to proteolytic degradation, pH-activity profiles and the like.

A change in substrate specificity is defined as a difference between the kcat/Km ratio of the precursor carbonyl hydrolase and that of the hydrolase mutant. The kcat/Km ratio is a measure of catalytic efficienty. Carbonyl hydrolase mutants with increased or diminished kcat/Km ratios are described in the examples. Generally, the objective will be to secure a mutant having a greater (numerically large) kcat/Km ratio for a given substrate, thereby enabling the use of the enzyme to more efficiently act on a target substrate. A substantial change in kcat/Km ratio is preferably at least 2-fold increase or decrease. However, smaller increases or decreases in the ratio (e.g., at least 1.5-fold) are also considered substantial. An increase in kcat/Km ratio for one substrate may be accompanied by a reduction in kcat/Km ratio for another substrate. This is a shift in substrate specificity, and mutants exhibiting such shifts have utility where the precursor hydrolase is undesirable, e.g. to prevent undesired hydrolysis of a particular substrate in an admixture of substrates. Km and kcat are measured in accord with known procedures, as described in EPO Publication No. 0130756 or as described herein.

Oxidative stability is measured either by known procedures or by the methods described hereinafter. A substantial change in oxidative stability is evidenced by at least about 50% increase or decrease (preferably decrease) in the rate of loss of enzyme activity when exposed to various oxidizing conditions. Such oxidizing conditions are exposure to the organic oxidant diperdodecanoic acid (DPDA) under the conditions described in the examples.

Alkaline stability is measured either by known procedures or by the methods described herein. A substantial change in alkaline stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half life of the enzymatic activity of a mutant when compared to the precursor carbonyl hydrolase. In the case of subtilisins, alkaline stability was measured as a function of autoproteolytic degradation of subtilisin at alkaline pH, e.g. for example, 0.1M sodium phosphate, pH 12 at 25° or 30°C.

Thermal stability is measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half-life of the catalytic activity of a mutant when exposed to a relatively high temperature and neutral pH as compared to the precursor carbonyl hydrolase. In the case of subtilisins, thermal stability is measured by the autoproteolytic degradation of subtilisin at elevated temperatures and neutral pH, e.g., for example 2mM calcium chloride, 50mM MOPS pH 7.0 at 59°C.

The inventors have produced mutant subtilisins containing the substitution of the amino acid residues of B. amyloliquefaciens subtilisin shown in Table I. The wild type amino acid sequence and DNA sequence of B. amyloliquefaciens subtilisin is shown in Fig. 1.

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TABLE I

Residue	Replacement Amino Acid
Tyr21	F A
Thr22	C
Ser24	C
Asp32	Q S
Ser33	A T
Asp36	A G
Gly46	V
Ala48	E V R
Ser49	C L
Met50	C F V
Asn77	D
Ser87	C
Lys94	C
Val95	C
Leu96	D
Tyr104	A C D E F G H I K L M N P Q R S T V W
Ile107	V
Gly110	C R
Met124	I L
Asn155	A D H Q T
Glu156	Q S
Gly166	C E I L M P S T W Y
Gly169	C D E F H I K L M N P Q R T V W Y
Lys170	E R
Tyr171	F
Pro172	E Q
Phe189	A C D E G H I K L M N P Q R S T V W Y
Asp197	R A
Met199	I
Ser204	C R L P
Lys213	R T
Tyr217	A C D E F G H I K L M N P Q R S T V W
Ser221	A C

The different amino acids substituted are represented in Table I by the following single letter designations:

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Amino acid or residue thereof	3-letter symbol	1-letter symbol
Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H

Except where otherwise indicated by context, wild-type amino acids are represented by the above three-letter symbols and replaced amino acids by the above single-letter symbols. Thus, if the methionine at residue 50 in B. amyloliquefaciens subtilisin is replaced by phenylalanine, this mutation (mutant) may be designated Met50F or F50. Similar designations are used for multiple mutants.

In addition to the amino acids used to replace the residues disclosed in Table I, other replacements of amino acids at these residues are expected to produce mutant subtilisins having useful properties. These residues and replacement amino acids are shown in Table II.

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TABLE II

Residue	Replacement Amino Acid(s)
Tyr-21	L
Thr22	K
Ser24	A
Asp32	
Ser33	G
Gly46	
Ala48	
Ser49	
Met50	L K I V
Asn77	D
Ser87	N
Lys94	R Q
Val95	L I
Tyr104	
Met124	K A
Ala152	C L I T M
Asn155	
Glu156	A T M L Y
Gly166	
Gly169	
Tyr171	K R E Q
Pro172	D N
Phe189	
Tyr217	
Ser221	
Met222	

Each of the mutant subtilisins in Table I contain the replacement of a single residue of the B. amyloliquefaciens amino acid sequence. These particular residues were chosen to probe the influence of such substitutions on various properties of B. amyloliquefacien subtilisin.

Thus, the inventors have identified Met124 and Met222 as important residues which if substituted with another amino acid produce a mutant subtilisin with enhanced oxidative stability. For Met124, Leu and Ile are preferred replacement amino acids. Preferred amino acids for replacement of Met222 are disclosed in EPO Publication No. 0130756.

Various other specific residues have also been identified as being important with regard to substrate specificity. These residues include Tyr104, Ala152, Glu156, Gly166, Gly169, Phe189 and Tyr217 for which mutants containing the various replacement amino acids presented in Table I have already been made, as well as other residues presented below for which mutants have yet to be made.

The identification of these residues, including those yet to be mutated, is based on the inventors' high resolution crystal structure of B. amyloliquefaciens subtilisin to 1.8 A (see Table III), their experience with in vitro mutagenesis of subtilisin and the literature on subtilisin. This work and the x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449), product complexes (Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), and transition state analogs (Matthews, D.A., et al (1975) J. Biol. Chem. 250, 7120-7126; Poulos, T.L., et al. (1976) J. Biol. Chem. 251, 1097-1103), has helped in identifying an extended peptide binding cleft in subtilisin. This substrate binding cleft together with substrate is schematically diagramemed in Fig. 2, according to the nomenclature of Schechter, I., et al. (1967) Biochem Bio. Res. Commun. 27, 157. The scissile bond in the substrate is identified by an arrow. The P and P' designations refer to the amino acids which are positioned respectively toward the amino or carboxy terminus relative to the scissle bond. The S and S' designations refer to subsites in the substrate binding cleft of subtilisin which interact with the corresponding substrate amino acid residues.

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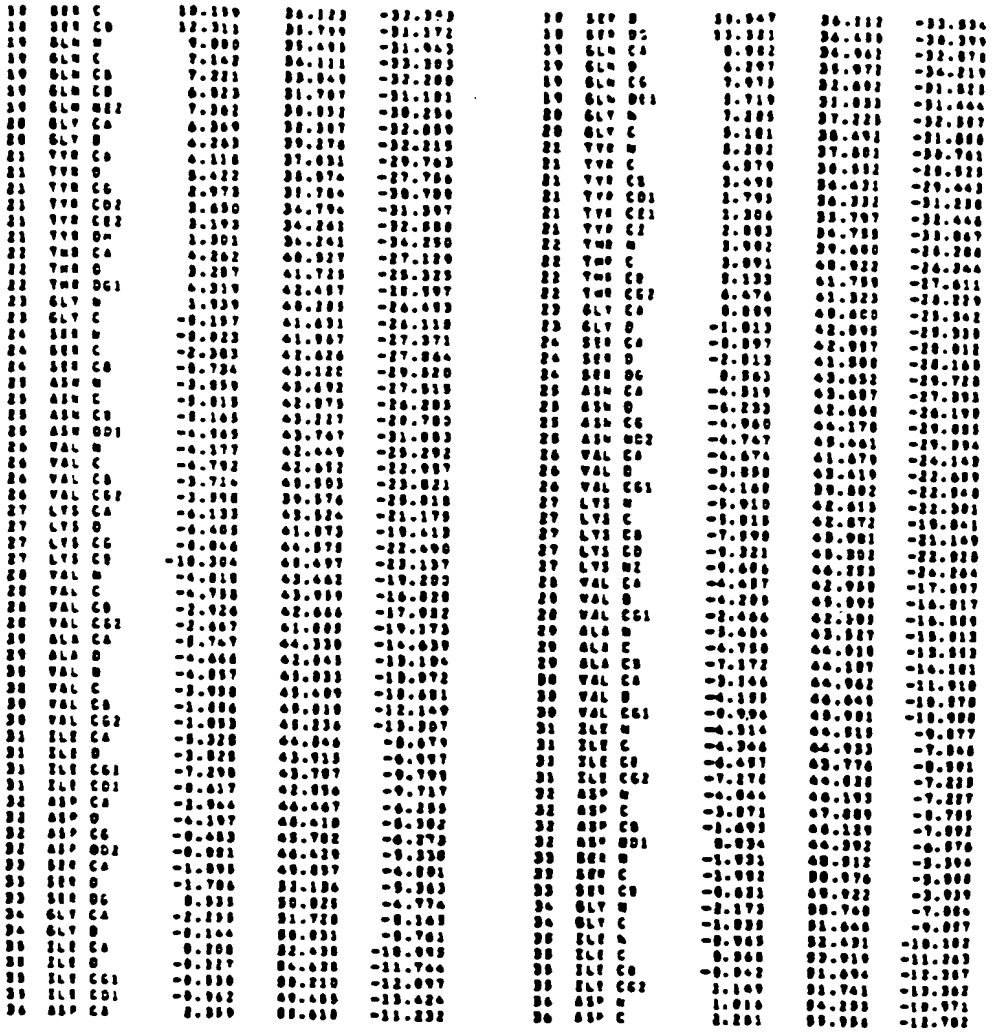
Atomic Coordinates for the
Apoenzyme Form of B. Amyloliquefaciens
Subtilisin to 1.8AResolution

1	ALA	N	39.434	53.195	-21.756	1	ALA	CA	39.911	51.774	-21.965
1	ALA	C	38.731	50.955	-21.324	1	ALA	O	38.374	51.197	-20.175
1	ALA	CB	23.899	51.518	-21.383	2	GLN	N	38.268	49.806	-22.043
2	GLN	CA	37.219	49.808	-21.434	2	GLN	C	37.875	47.706	-20.992
2	GLN	O	38.765	47.165	-21.691	2	GLN	CB	36.125	48.760	-22.449
2	GLN	CG	35.328	47.885	-21.927	2	GLN	CD	35.912	47.762	-22.930
2	GLN	OE1	33.023	48.612	-22.867	2	GLN	NE2	34.115	46.917	-23.926
3	SER	N	37.477	47.205	-19.852	3	SER	CA	37.958	45.868	-19.437
3	SER	C	36.735	44.938	-19.498	3	SER	O	35.598	45.352	-19.229
3	SER	CB	38.588	45.038	-18.869	3	SER	OG	37.682	46.218	-17.849
4	VAL	N	36.991	43.646	-19.725	4	VAL	CA	35.946	42.619	-19.639
4	VAL	C	36.129	41.934	-18.298	4	VAL	O	37.123	41.178	-18.886
4	VAL	CB	34.808	43.822	-20.822	4	VAL	CG1	34.874	40.572	-20.741
4	VAL	CG2	36.037	42.266	-22.184	5	PRO	N	35.239	42.184	-17.331
5	PRO	CA	35.384	41.415	-16.827	5	PRO	C	35.501	39.985	-16.249
5	PRO	O	34.885	39.263	-17.146	5	PRO	CB	34.350	41.880	-15.263
5	PRO	CG	33.641	43.215	-15.921	5	PRO	CD	34.844	42.986	-17.617
6	TYR	N	36.363	39.240	-15.487	6	TYR	CA	36.628	37.883	-15.715
6	TYR	C	35.359	36.975	-15.528	6	TYR	O	35.224	35.943	-16.235
6	TYR	CB	37.824	37.823	-14.834	6	TYR	CG	38.821	33.847	-15.855
6	TYR	CD1	38.637	35.652	-14.346	6	TYR	CD2	37.696	34.988	-14.971
6	TYR	CE1	38.535	34.870	-14.653	6	TYR	CE2	37.815	33.539	-14.379
6	TYR	CZ	38.222	33.154	-15.428	6	TYR	OH	38.312	31.838	-15.996
7	GLY	N	34.464	37.362	-14.630	7	GLY	CA	33.231	36.648	-14.376
7	GLY	C	32.488	36.535	-15.670	7	GLY	O	31.747	35.478	-15.883
8	VAL	N	32.441	37.529	-16.541	8	VAL	CA	31.777	37.523	-17.636
8	VAL	C	32.363	36.433	-18.735	8	VAL	O	31.639	35.716	-19.470
8	VAL	CB	31.765	38.900	-18.567	8	VAL	CG1	31.186	38.893	-19.963
8	VAL	CG2	30.991	39.919	-17.733	9	SER	N	33.661	36.318	-18.775
9	SER	CA	34.419	35.342	-19.562	9	SER	C	34.188	33.928	-18.965
9	SER	O	34.112	33.814	-19.801	9	SER	CB	35.926	35.632	-19.585
9	SER	OG	36.162	36.767	-20.358	10	GLN	N	34.115	33.887	-17.662
10	GLN	CA	33.964	32.636	-18.876	10	GLN	C	32.687	31.887	-17.277
10	GLN	O	32.785	30.642	-17.613	10	GLN	CB	34.125	32.885	-15.418
10	GLN	CG	34.295	31.617	-14.588	10	GLN	CD	34.486	31.911	-13.167
10	GLN	OE1	34.554	33.068	-12.744	10	GLN	NE2	34.552	30.960	-12.251
11	ILE	N	31.625	32.575	-17.670	11	ILE	CA	30.373	31.984	-16.102
11	ILE	C	30.209	31.792	-19.695	11	ILE	O	9.173	31.333	-20.180
11	ILE	CB	9.132	32.669	-17.675	11	ILE	CG1	9.066	34.117	-16.849
11	ILE	CG2	9.162	32.655	-15.941	11	ILE	CD1	7.588	34.648	-17.923
12	LYS	N	31.272	32.185	-20.277	12	LYS	CA	31.388	32.119	-21.722
12	LYS	C	30.456	33.006	-22.522	12	LYS	O	30.178	32.783	-23.606
12	LYS	CB	31.257	30.646	-22.216	12	LYS	CG	32.283	29.838	-21.423
12	LYS	CD	32.563	28.517	-22.159	12	LYS	CE	33.023	27.467	-21.164
12	LYS	NZ	34.476	27.680	-20.935	13	ALA	N	30.189	34.138	-21.991
13	ALA	CA	9.325	35.198	-22.631	13	ALA	C	30.826	35.716	-23.865
13	ALA	O	9.338	35.804	-24.983	13	ALA	CB	8.865	36.195	-21.565
14	PRO	N	31.332	35.930	-23.893	14	PRO	CA	31.985	36.430	-25.120
14	PRO	C	31.706	35.957	-26.317	14	PRO	O	31.778	36.867	-27.445
14	PRO	CB	33.462	36.588	-24.692	14	PRO	CG	33.328	36.978	-23.271
14	PRO	CD	32.281	35.936	-22.758	15	ALA	N	31.540	36.236	-26.129
15	ALA	CA	31.379	33.458	-27.367	15	ALA	C	30.882	33.795	-28.832
15	ALA	O	30.009	33.718	-29.278	15	ALA	CB	31.982	31.969	-27.062
16	LEU	N	9.883	34.138	-27.240	16	LEU	CA	7.791	34.558	-27.828
16	LEU	C	7.912	31.925	-28.521	16	LEU	O	7.342	30.126	-29.188
16	LEU	CB	6.766	34.673	-26.698	16	LEU	CG	5.798	33.665	-26.822
16	LEU	CD1	5.881	33.234	-27.809	16	LEU	CD2	6.694	32.287	-26.283
17	HIS	N	8.665	36.820	-27.922	17	HIS	CA	8.898	38.191	-28.530
17	HIS	C	9.518	37.981	-29.890	17	HIS	O	9.187	38.622	-30.856
17	HIS	CB	9.788	39.188	-27.652	17	HIS	CG	9.185	39.288	-26.262
17	HIS	CD1	9.938	39.887	-25.272	17	HIS	CD2	8.888	38.926	-25.694
17	HIS	CE1	9.226	39.914	-24.164	17	HIS	NE2	8.879	39.328	-24.381
18	SER	N	10.448	37.833	-30.822	18	SER	CA	11.189	38.739	-31.322



36	ASP N	3.084	55.471	-13.579	36	ASP CB	3.712	55.720	-10.514
36	ASP CG	4.339	57.099	-10.886	36	ASP OD1	3.755	57.974	-11.429
36	ASP OD2	5.448	57.277	-10.263	37	SER N	1.304	56.822	-13.111
37	SER CA	1.183	57.221	-14.512	37	SER C	2.377	58.095	-14.969
37	SER O	2.545	58.303	-16.151	37	SER CB	-0.093	58.069	-14.788
37	SER OG	-0.088	59.133	-13.079	38	SER N	3.163	58.614	-14.881
38	SER CA	4.261	59.505	-14.487	38	SER C	5.466	58.705	-14.992
38	SER O	6.543	59.251	-15.205	38	SER CB	4.742	60.435	-13.398
38	SER OG	5.316	59.865	-12.234	39	HIS N	5.454	57.390	-14.892
39	HIS CA	6.637	56.576	-15.291	39	HIS C	6.681	56.481	-16.778
39	HIS O	5.738	55.078	-17.619	39	HIS CB	6.637	55.203	-14.515
39	HIS CG	8.014	54.609	-14.656	39	HIS ND1	8.795	54.356	-15.561
39	HIS CD2	8.769	54.345	-13.389	39	HIS CE1	9.970	53.938	-15.130
39	HIS NE2	9.986	53.918	-13.808	40	PRO N	7.887	56.836	-17.387
40	PRO CA	7.988	56.697	-18.031	40	PRO C	8.156	55.288	-19.357
40	PRO O	8.032	55.097	-20.570	40	PRO CB	9.247	57.533	-19.161
40	PRO CG	10.833	57.405	-17.902	40	PRO CD	8.988	57.452	-16.776
41	ASP N	8.681	54.328	-18.485	41	ASP OD2	11.140	50.399	-18.668
41	ASP OD1	10.325	52.395	-20.429	41	ASP CG	10.473	51.387	-19.211
41	ASP CB	9.799	52.239	-18.224	41	ASP CA	8.665	52.959	-18.966
41	ASP C	7.311	52.163	-18.839	41	ASP O	7.396	50.947	-18.977
42	LEU N	6.185	52.803	-18.550	42	LEU CA	4.892	52.147	-18.466
42	LEU C	3.924	52.907	-19.376	42	LEU O	3.993	54.163	-19.490
42	LEU CB	6.421	52.158	-17.008	42	LEU CG	5.182	51.363	-15.946
42	LEU CD1	4.535	51.546	-16.581	42	LEU CD2	5.273	49.877	-16.350
43	LYS N	3.018	52.135	-19.946	43	LYS CA	1.893	52.605	-20.721
43	LYS C	0.637	52.156	-20.818	43	LYS O	0.504	50.920	-19.820
43	LYS CB	2.021	52.389	-22.169	43	LYS CG	0.685	52.436	-22.910
43	LYS CD	0.998	52.862	-24.339	43	LYS CE	-0.180	52.504	-25.260
43	LYS NZ	0.337	51.757	-26.418	44	VAL N	-0.191	53.035	-19.490
44	VAL CA	-1.607	52.639	-18.765	44	VAL C	-2.571	52.807	-19.731
44	VAL O	-2.623	53.906	-20.634	44	VAL CB	-1.480	53.351	-17.383
44	VAL CG1	-2.724	52.941	-16.502	44	VAL CG2	-0.197	53.194	-16.553
45	ALA N	-3.494	51.951	-19.871	45	ALA CA	-4.619	51.977	-20.810
45	ALA C	-5.841	52.507	-20.053	45	ALA O	-6.703	53.085	-20.703
45	ALA CB	-4.031	50.500	-21.389	46	GLY N	-5.910	52.356	-18.768
46	GLY CA	-7.082	52.837	-18.081	46	GLY C	-6.987	52.443	-16.538
46	GLY O	-5.938	52.806	-16.035	47	GLY N	-8.092	52.658	-15.793
47	GLY CA	-8.016	52.246	-14.388	47	GLY C	-9.179	52.757	-13.572
47	GLY O	-9.988	53.481	-14.185	48	ALA N	-9.221	52.446	-12.338
48	ALA CA	-10.235	52.070	-11.382	48	ALA C	-9.790	52.675	-9.968
48	ALA O	-9.846	51.720	-9.725	48	ALA CB	-11.558	52.100	-11.617
49	SER N	-10.149	53.547	-9.037	49	SER CA	-9.752	53.355	-7.652
49	SER C	-10.947	52.986	-6.783	49	SER O	-11.972	53.677	-6.908
49	SER CB	-9.092	54.508	-7.029	49	SER OG	-8.879	54.255	-5.658
50	MET N	-10.835	52.087	-5.932	50	MET CA	-11.052	51.569	-4.974
50	MET C	-11.463	51.962	-3.561	50	MET O	-11.997	51.398	-2.575
50	MET CB	-12.012	50.018	-4.996	50	MET CG	-11.912	49.463	-6.389
50	MET SD	-13.460	49.889	-7.256	50	MET CE	-12.088	50.111	-8.903
51	VAL N	-10.427	52.760	-3.422	51	VAL CA	-9.968	53.170	-2.067
51	VAL C	-10.630	54.562	-1.907	51	VAL O	-10.237	55.437	-2.682
51	VAL CB	-8.643	53.155	-2.000	51	VAL CG1	-7.892	53.579	-0.631
51	VAL CG2	-7.764	51.815	-2.302	52	PRO N	-11.621	54.693	-1.056
52	PRO CA	-12.372	55.933	-0.821	52	PRO C	-11.490	57.123	-0.440
52	PRO O	-11.771	58.220	-0.925	52	PRO CB	-13.488	55.594	0.244
52	PRO CG	-13.503	54.103	0.005	52	PRO CD	-12.264	53.620	-0.175
53	SER N	-10.642	56.986	0.299	53	SER CA	-9.538	57.982	0.682
53	SER C	-8.420	58.265	-0.326	53	SER O	-7.679	59.224	-0.038
53	SER CB	-9.004	57.707	2.069	53	SER OG	-8.256	56.521	2.127
54	GLU N	-8.254	57.523	-1.393	54	GLU CA	-7.204	57.668	-2.621
54	GLU C	-7.767	57.303	-3.785	54	GLU O	-7.533	56.243	-6.379
54	GLU CB	-6.134	56.599	-2.154	54	GLU CG	-5.289	56.959	-0.927
54	GLU CD	-6.866	54.967	-0.870	54	GLU OE1	-5.545	55.694	-1.968

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54	GLN	HE2	-3.990	55.777	0.271	53	THR	O	-0.571	50.251	-4.249
55	THR	CA	-9.433	58.121	-5.441	53	THR	C	-0.764	50.139	-6.779
55	THR	O	-9.633	57.919	-7.010	53	THR	CB	-10.986	49.208	-5.383
55	THR	OG1	-9.885	60.110	-5.418	53	THR	CG2	-11.432	49.163	-4.011
56	ASN	N	-7.482	58.403	-6.877	54	ASN	HD2	-6.938	41.170	-9.881
56	ASN	OD1	-5.875	58.967	-10.337	54	ASN	CG	-5.273	50.925	-9.355
56	ASN	CB	-5.898	59.494	-8.288	54	ASN	CA	-6.762	50.425	-8.288
56	ASN	C	-6.812	57.994	-8.305	56	ASN	O	-5.104	56.866	-7.478
57	PRO	N	-6.362	56.261	-9.258	57	PRO	CG	-7.123	55.257	-11.177
57	PRO	CD	-7.584	56.433	-10.272	57	PRO	CB	-6.644	54.178	-10.235
57	PRO	CA	-5.679	54.941	-9.532	57	PRO	C	-4.381	55.882	-9.946
57	PRO	O	-3.509	56.128	-9.965	58	PHE	N	-3.998	56.262	-10.491
58	PHE	CA	-2.747	54.877	-11.222	58	PHE	C	-1.712	57.129	-10.253
58	PHE	O	-0.635	57.497	-10.688	58	PHE	CG	-2.943	57.582	-12.423
58	PHE	CG	-3.983	56.968	-13.357	58	PHE	CD1	-3.756	55.788	-14.059
58	PHE	CD2	-5.211	57.638	-13.459	58	PHE	CE1	-4.722	55.255	-14.928
58	PHE	CE2	-6.196	57.095	-14.276	58	PHE	CZ	-5.949	55.939	-15.051
59	GLN	N	-2.044	57.339	-8.998	59	GLN	CA	-1.172	57.583	-7.934
59	GLN	C	-0.807	56.481	-7.008	59	GLN	O	-1.439	56.883	-6.115
59	GLN	CB	-1.662	58.668	-7.889	59	GLN	CG	-0.962	59.261	-6.836
59	GLN	CD	-1.798	60.157	-5.158	59	GLN	OE1	-1.404	61.288	-6.836
59	GLN	NE2	-2.959	59.485	-6.742	60	ASP	N	0.410	55.895	-7.211
60	ASP	CA	0.851	54.792	-6.304	60	ASP	C	1.631	55.267	-5.090
60	ASP	O	2.827	55.550	-5.231	60	ASP	CB	1.396	53.744	-7.188
60	ASP	CG	2.077	52.538	-6.380	60	ASP	OD1	1.766	52.337	-5.190
60	ASP	OD2	2.915	51.841	-7.030	61	ASN	N	0.959	55.265	-3.958
61	ASN	HD2	-1.364	57.747	-2.347	61	ASN	OD1	0.666	58.566	-2.875
61	ASN	CG	-0.840	57.670	-2.399	61	ASN	CB	0.531	56.401	-1.784
61	ASN	CA	1.557	55.734	-2.788	61	ASN	C	2.291	54.632	-1.968
61	ASN	O	2.933	54.862	-0.982	62	ASN	N	2.218	53.634	-2.468
62	ASN	CA	2.877	52.348	-1.789	62	ASN	C	4.124	51.893	-2.679
62	ASN	O	4.951	51.313	-1.778	62	ASN	CB	1.783	51.319	-1.621
62	ASN	CG	2.371	50.183	-0.697	62	ASN	OD1	2.633	49.877	-1.343
62	ASN	HD2	2.422	50.208	0.601	63	SER	N	4.152	52.104	-3.761
63	SER	CA	5.189	51.696	-4.789	63	SER	C	5.871	50.256	-5.289
63	SER	O	5.593	49.790	-6.269	63	SER	CB	6.523	51.958	-4.812
63	SER	OG	6.871	50.698	-3.418	64	HIS	N	4.282	49.675	-4.639
64	HIS	CA	3.994	48.859	-4.935	64	HIS	C	3.366	47.759	-6.261
64	HIS	O	3.861	46.974	-7.108	64	HIS	CB	3.184	47.581	-3.747
64	HIS	CG	3.144	46.021	-3.726	64	HIS	HD1	2.187	45.247	-4.241
64	HIS	CD2	4.856	45.194	-3.135	64	HIS	CE1	2.416	43.966	-4.854
64	HIS	HE2	3.556	43.920	-3.368	65	GLY	N	2.287	48.628	-6.587
65	GLY	CA	1.552	48.264	-7.838	65	GLY	C	2.392	48.636	-9.037
65	GLY	O	2.239	48.078	-10.134	66	THR	N	3.233	49.659	-8.832
66	THR	CA	4.864	50.117	-9.954	66	THR	C	5.889	49.809	-10.291
66	THR	O	5.333	48.789	-11.461	66	THR	CB	4.744	51.511	-9.667
66	THR	OG1	3.637	52.425	-9.406	66	THR	CG2	5.936	52.078	-10.849
67	HIS	N	5.685	48.443	-9.276	67	HIS	CA	6.783	47.341	-9.458
67	HIS	C	6.891	46.141	-10.143	67	HIS	O	6.849	45.436	-11.150
67	HIS	CB	7.388	47.871	-8.064	67	HIS	CG	8.595	46.275	-8.148
67	HIS	HD1	8.398	44.907	-8.276	67	HIS	CD2	9.984	46.678	-8.076
67	HIS	CE1	9.857	44.491	-8.299	67	HIS	HE2	10.678	45.514	-8.186
68	VAL	N	6.892	45.749	-9.731	68	VAL	CA	6.142	44.687	-10.266
68	VAL	C	3.856	44.868	-11.748	68	VAL	O	6.116	43.942	-12.535
68	VAL	CB	2.939	44.252	-9.384	68	VAL	CG1	3.960	43.268	-10.829
68	VAL	CG2	3.319	43.785	-8.888	69	ALA	N	3.373	46.869	-12.113
69	ALA	CA	3.837	46.468	-13.429	69	ALA	C	6.193	46.398	-14.611
69	ALA	O	4.028	45.913	-15.965	69	ALA	CB	3.332	47.851	-13.386
70	GLY	N	5.368	46.782	-13.916	70	GLY	CA	6.595	46.805	-14.670
70	GLY	C	7.846	45.378	-15.821	70	GLY	O	7.606	45.156	-16.119
71	THR	N	6.828	44.431	-14.138	71	THR	CA	7.177	43.819	-14.446
71	THR	C	6.224	42.596	-15.543	71	THR	O	6.882	43.820	-16.495
71	THR	CB	7.119	42.878	-13.191	71	THR	OG1	8.191	42.592	-12.390

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71	THR	CG2	7.274	40.983	-19.396	72	VAL	N	6.938	42.887	-19.427
72	VAL	CA	3.976	42.491	-16.484	72	VAL	C	4.312	43.884	-17.831
72	VAL	N	4.341	42.388	-18.868	72	VAL	CB	2.916	42.867	-16.885
72	VAL	CG1	1.512	42.488	-17.178	72	VAL	CG2	2.142	42.327	-14.723
73	ALA	N	4.584	44.417	-17.988	73	ALA	CA	4.587	45.891	-18.167
73	ALA	C	5.433	46.333	-19.355	73	ALA	O	5.062	47.188	-20.216
73	ALA	CB	3.107	45.441	-19.433	74	ALA	N	6.944	46.429	-18.635
74	ALA	CA	7.478	47.591	-18.959	74	ALA	C	7.748	47.648	-20.342
74	ALA	N	7.959	46.648	-21.854	74	ALA	CB	8.653	47.446	-17.925
75	LEU	N	7.458	48.784	-21.839	75	LEU	CA	7.812	48.968	-22.456
75	LEU	C	9.192	48.568	-22.966	75	LEU	O	10.162	48.758	-22.253
75	LEU	CB	7.548	50.471	-22.809	75	LEU	CG	6.123	50.913	-22.379
75	LEU	CD1	6.879	52.436	-22.388	75	LEU	CD2	5.896	50.662	-23.405
76	ASN	N	9.167	48.103	-26.169	76	ASN	OD2	22.385	46.432	-26.304
76	ASN	OD1	10.958	45.848	-27.928	76	ASN	CG	11.195	46.276	-26.882
76	ASN	CB	10.818	46.651	-25.988	76	ASN	CA	10.359	47.738	-24.938
76	ASN	C	10.783	49.868	-25.643	76	ASN	O	10.357	49.479	-26.619
77	ASN	N	11.804	49.664	-23.871	77	ASN	CA	12.220	50.957	-25.681
77	ASN	C	13.787	51.829	-25.348	77	ASN	O	14.364	49.979	-23.313
77	ASN	CB	11.335	52.076	-25.117	77	ASN	CG	11.258	52.827	-23.616
77	ASN	OD1	12.032	51.346	-22.917	77	ASN	ND2	10.296	52.741	-23.825
78	SER	N	14.125	52.267	-25.164	78	SER	CA	15.513	52.614	-24.906
78	SER	C	15.810	52.742	-23.436	78	SER	O	16.982	53.071	-23.166
78	SER	CB	15.905	53.941	-25.587	78	SER	OG	15.926	53.878	-26.999
79	ILE	N	14.858	52.565	-22.529	79	ILE	CA	15.155	52.784	-21.120
79	ILE	C	14.617	51.683	-20.230	79	ILE	O	13.843	50.841	-20.679
79	ILE	CB	14.471	54.174	-20.697	79	ILE	CG1	12.965	54.032	-20.814
79	ILE	CG2	14.997	55.328	-21.612	79	ILE	CD1	12.135	55.176	-20.155
80	GLY	N	14.995	51.768	-18.981	80	GLY	CA	14.476	50.948	-17.913
80	GLY	C	14.612	49.448	-18.219	80	GLY	O	15.719	48.994	-18.544
81	VAL	N	13.513	48.766	-17.988	81	VAL	CA	13.611	47.286	-19.061
81	VAL	C	12.511	46.919	-19.217	81	VAL	O	12.260	47.739	-20.117
81	VAL	CB	13.881	46.755	-16.677	81	VAL	CG1	14.038	47.086	-15.573
81	VAL	CG2	11.638	47.261	-16.231	82	LEU	N	12.126	48.645	-19.216
82	LEU	CA	11.312	45.828	-20.256	82	LEU	C	10.398	44.928	-19.510
82	LEU	O	10.456	43.336	-18.688	82	LEU	CB	12.286	44.259	-21.229
82	LEU	CG	11.430	43.568	-22.366	82	LEU	CD1	10.796	44.657	-23.223
82	LEU	CD2	12.359	42.675	-23.192	83	GLY	N	9.131	44.188	-19.016
83	GLY	CA	8.133	43.328	-19.184	83	GLY	C	8.027	42.011	-19.925
83	GLY	O	8.946	41.822	-21.026	84	VAL	N	7.272	41.112	-19.283
84	VAL	CA	6.973	39.807	-19.888	84	VAL	C	6.164	40.038	-21.148
84	VAL	O	6.426	39.472	-22.194	84	VAL	CB	6.256	38.920	-18.841
84	VAL	CG1	5.680	37.677	-19.557	84	VAL	CG2	7.190	38.807	-17.785
85	ALA	N	5.156	40.926	-21.024	85	ALA	CA	4.217	41.194	-22.158
85	ALA	C	4.213	42.683	-22.396	85	ALA	O	3.268	43.401	-22.838
85	ALA	CB	2.846	40.663	-21.748	86	PRO	N	5.248	43.186	-23.059
86	PRO	CA	5.413	44.635	-23.285	86	PRO	C	4.321	45.371	-23.947
86	PRO	O	4.291	46.695	-23.849	86	PRO	CB	6.822	44.784	-23.813
86	PRO	CG	7.838	43.464	-24.546	86	PRO	CD	6.377	42.448	-23.636
87	SER	N	3.548	44.476	-24.769	87	SER	CA	2.689	45.324	-25.529
87	SER	C	1.193	45.132	-24.897	87	SER	O	0.162	45.813	-25.619
87	SER	CB	2.401	44.777	-26.927	87	SER	OG	3.591	45.143	-27.583
88	ALA	N	1.817	44.566	-23.762	88	ALA	CB	-0.163	43.518	-21.828
88	ALA	CA	-0.273	44.353	-23.884	88	ALA	C	-0.898	45.717	-22.690
88	ALA	O	-0.174	46.719	-22.435	89	SER	N	-2.219	45.691	-22.678
89	SER	OG	-0.146	47.182	-24.288	89	SER	CB	-4.343	46.983	-22.899
89	SER	CA	-3.981	46.867	-22.227	89	SER	C	-3.136	46.788	-20.727
89	SER	N	-3.793	45.844	-20.209	90	LEU	N	-2.446	47.656	-20.037
90	LEU	CA	-2.378	47.667	-18.593	90	LEU	C	-3.483	48.438	-17.804
90	LEU	O	-3.582	49.604	-18.215	90	LEU	CB	-0.951	48.273	-18.476
90	LEU	CG	-0.233	47.851	-17.194	90	LEU	CD1	-0.876	46.361	-17.219
90	LEU	CD2	1.168	48.524	-17.847	91	TYR	N	-4.264	47.964	-16.938
91	TYR	CA	-5.258	48.678	-16.137	91	TYR	C	-4.873	48.758	-14.685

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91	TYR	B	-4.494	47.749	-14.023	91	TYR	CB	-4.486	48.993	-14.314
91	TYR	CG	-7.894	48.237	-17.741	91	TYR	CD1	-4.595	47.415	-18.755
91	TYR	CD2	-7.971	49.275	-18.169	91	TYR	CE1	-4.985	47.872	-20.096
91	TYR	CE2	-8.315	49.421	-19.492	91	TYR	CZ	-7.796	48.882	-20.463
91	TYR	OH	-8.182	48.752	-21.764	92	ALA	N	-4.895	49.958	-14.184
92	ALA	CA	-4.949	50.199	-12.787	92	ALA	C	-5.823	50.833	-11.983
92	ALA	O	-6.723	50.898	-12.858	92	ALA	CB	-3.997	51.621	-12.488
93	VAL	N	-5.959	48.993	-11.129	93	VAL	CA	-7.183	48.854	-10.325
93	VAL	C	-6.788	49.814	-8.899	93	VAL	O	-6.181	47.993	-8.372
93	VAL	CB	-7.957	47.555	-10.621	93	VAL	CG1	-9.213	47.488	-9.725
93	VAL	CG2	-8.195	47.378	-12.072	94	LYS	N	-6.987	50.217	-8.327
94	LYS	CA	-6.378	50.464	-6.999	94	LYS	C	-7.331	49.985	-5.894
94	LYS	O	-8.458	50.480	-5.783	94	LYS	CB	-6.051	51.976	-6.818
94	LYS	CG	-5.396	52.320	-5.447	94	LYS	CD	-4.868	53.785	-5.582
94	LYS	CE	-4.399	54.288	-4.199	94	LYS	NZ	-3.735	55.944	-4.387
95	VAL	N	-6.909	48.871	-5.076	95	VAL	CA	-7.646	48.457	-3.920
95	VAL	C	-6.919	48.699	-2.568	95	VAL	O	-7.425	48.156	-1.581
95	VAL	CB	-8.184	47.030	-4.319	95	VAL	CG1	-8.868	46.852	-5.619
95	VAL	CG2	-6.988	46.188	-4.332	96	LEU	N	-5.676	48.976	-2.604
96	LEU	CA	-6.782	49.193	-1.486	96	LEU	C	-6.331	50.559	-1.321
96	LEU	O	-3.942	51.121	-2.336	96	LEU	CB	-3.589	48.241	-1.573
96	LEU	CG	-3.593	46.799	-2.072	96	LEU	CD1	-2.287	46.184	-2.163
96	LEU	CD2	-4.489	46.882	-1.845	97	GLY	N	-4.326	50.975	-0.886
97	GLY	CA	-3.898	52.387	0.287	97	GLY	C	-2.363	52.437	0.385
97	GLY	O	-1.619	51.463	0.165	98	ALA	N	-1.954	53.648	0.758
98	ALA	CB	-0.428	55.478	1.518	98	ALA	CA	-0.563	54.068	0.965
98	ALA	C	0.180	53.138	1.917	98	ALA	O	1.393	52.921	1.443
99	ASP	N	-0.506	52.573	2.912	99	ASP	OD2	-2.631	51.042	4.151
99	ASP	OD1	-2.738	50.902	4.083	99	ASP	CG	-2.083	51.131	3.040
99	ASP	CB	-0.648	51.603	3.175	99	ASP	CA	0.101	51.618	3.055
99	ASP	C	0.146	50.165	3.328	99	ASP	O	0.735	49.313	4.029
100	GLY	N	-0.424	49.883	2.168	100	GLY	CA	-0.343	48.523	1.615
100	GLY	C	-1.520	47.651	2.882	100	GLY	O	-1.649	46.512	1.479
101	SER	N	-2.342	48.128	2.998	101	SER	CA	-3.542	47.388	3.315
101	SER	C	-4.759	47.894	2.532	101	SER	O	-4.758	48.972	1.907
101	SER	CB	-3.716	47.447	4.817	101	SER	OG	-4.611	46.634	5.209
102	GLY	N	-5.821	47.892	2.577	102	GLY	CA	-7.077	47.422	1.896
102	GLY	C	-8.166	46.536	2.528	102	GLY	O	-7.888	45.431	3.838
103	GLN	N	-9.377	47.058	2.498	103	GLN	CA	-10.335	46.297	3.028
103	GLN	C	-10.963	45.232	2.822	103	GLN		-10.779	45.482	0.817
103	GLN	CB	-11.671	47.387	3.274	103	GLN	CG	-11.368	48.885	4.506
103	GLN	CD	-12.360	49.104	4.915	103	GLN	OE1	-12.159	49.816	5.902
103	GLN	NE2	-13.419	49.197	4.312	104	TYR	N	-11.611	44.161	2.451
104	TYR	CA	-12.868	43.326	1.506	104	TYR	C	-13.031	43.690	0.473
104	TYR	O	-12.939	43.276	-0.697	104	TYR	CB	-12.697	41.866	2.163
104	TYR	CG	-11.629	40.829	2.472	104	TYR	CD1	-11.819	39.789	3.377
104	TYR	CD2	-10.379	40.959	1.868	104	TYR	CE1	-10.809	38.885	3.787
104	TYR	CE2	-9.352	40.057	2.371	104	TYR	CZ	-9.564	39.022	3.081
104	TYR	OH	-8.481	38.191	3.324	105	SER	N	-13.989	44.572	0.983
105	SER	CA	-14.877	45.166	-0.834	105	SER	C	-14.172	45.928	-1.159
105	SER	O	-14.759	45.935	-2.258	105	SER	CB	-15.880	44.121	0.681
105	SER	OG	-15.209	47.039	1.458	106	TRP	N	-13.879	46.625	-0.836
106	TRP	CA	-12.421	47.391	-1.948	106	TRP	C	-11.895	46.436	-3.012
106	TRP	O	-12.871	46.648	-4.245	106	TRP	CB	-11.321	48.254	-1.355
106	TRP	CG	-11.645	49.111	-0.286	106	TRP	CD1	-12.062	49.524	0.744
106	TRP	CD2	-10.658	49.812	0.581	106	TRP	NE1	-12.691	50.358	1.340
106	TRP	CE2	-11.359	50.973	1.561	106	TRP	CE3	-9.275	49.852	0.576
106	TRP	CZ2	-10.671	51.318	2.588	106	TRP	CZ3	-8.468	50.583	1.525
106	TRP	CH2	-9.293	51.291	2.455	107	ILE	N	-11.339	45.338	-2.481
107	ILE	CA	-10.765	44.258	-3.325	107	ILE	C	-11.955	43.996	-4.198
107	ILE	O	-11.695	43.674	-5.398	107	ILE	CB	-9.966	43.183	-2.523
107	ILE	CG1	-8.636	43.784	-1.936	107	ILE	CG2	-9.632	41.930	-3.301
107	ILE	CD1	-8.263	42.998	-0.627	108	ILE	N	-12.994	43.292	-3.577

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100	ILE	CA	-14.136	42.722	-4.321	108	ILE	C	-14.439	43.694	-5.386
100	ILE	O	-14.894	43.320	-6.552	108	ILE	CB	-15.246	42.265	-3.320
100	ILE	CG1	-14.726	41.077	-2.482	108	ILE	CG2	-16.568	42.824	-4.095
100	ILE	CD1	-15.452	40.845	-1.131	109	ASN	N	-14.751	44.958	-4.981
109	ASN	CA	-15.284	46.818	-5.916	109	ASN	C	-14.232	46.867	-7.044
109	ASN	O	-14.660	46.272	-8.235	109	ASN	CB	-15.288	47.359	-5.287
109	ASN	CG	-16.528	47.486	-4.353	109	ASN	OD1	-17.495	46.695	-4.646
109	ASN	ND2	-16.633	48.667	-3.442	110	GLY	N	-12.951	45.988	-6.776
110	GLY	CA	-13.952	45.917	-7.865	110	GLY	C	-12.108	44.712	-8.812
110	GLY	O	-13.929	44.929	-10.036	111	ILE	N	-12.379	43.539	-8.246
111	ILE	CA	-12.603	42.334	-9.099	111	ILE	C	-13.059	42.360	-9.962
111	ILE	O	-13.921	42.384	-11.148	111	ILE	CB	-12.734	40.948	-8.364
111	ILE	CG1	-13.423	40.501	-7.655	111	ILE	CG2	-13.122	39.791	-9.367
111	ILE	CD1	-13.588	39.706	-6.336	112	GLU	N	-14.893	43.075	-9.288
112	GLU	CA	-16.118	43.376	-10.046	112	GLU	C	-15.872	44.367	-11.171
112	GLU	O	-16.467	44.130	-12.746	112	GLU	CB	-17.229	43.899	-9.141
112	GLU	CG	-17.847	42.917	-8.135	112	GLU	CD	-18.726	41.874	-8.685
112	GLU	OE1	-19.843	40.964	-8.816	112	GLU	OE2	-19.123	41.928	-9.866
113	TRP	N	-15.896	45.403	-10.971	113	TRP	CA	-16.756	46.488	-12.008
113	TRP	C	-16.876	45.663	-13.148	113	TRP	O	-16.319	45.932	-14.332
113	TRP	CB	-15.882	47.553	-11.436	113	TRP	CG	-15.486	48.336	-12.481
113	TRP	CD1	-16.148	49.736	-12.681	113	TRP	CD2	-12.441	48.552	-13.463
113	TRP	NE1	-15.597	50.443	-13.723	113	TRP	CE2	-12.545	49.761	-14.215
113	TRP	CE3	-11.451	47.645	-13.809	113	TRP	CZ2	-11.696	50.045	-15.274
113	TRP	CZ3	-10.610	47.899	-14.879	113	TRP	CH2	-10.752	49.874	-15.603
114	ALA	N	-13.089	44.881	-12.832	114	ALA	CA	-12.333	44.065	-13.874
114	ALA	C	-13.199	43.179	-14.752	114	ALA	O	-12.963	43.074	-15.978
114	ALA	CB	-11.299	43.192	-13.240	115	ILE	N	-14.174	42.560	-14.139
115	ILE	CA	-15.070	41.648	-14.897	115	ILE	C	-15.928	42.601	-15.856
115	ILE	O	-16.077	42.225	-17.078	115	ILE	CB	-16.089	40.048	-13.922
115	ILE	CG1	-15.218	39.036	-13.843	115	ILE	CG2	-17.151	40.168	-14.755
115	ILE	CD1	-16.004	39.611	-11.763	116	ALA	N	-16.534	43.527	-15.267
116	ALA	CA	-17.398	44.448	-16.050	116	ALA	C	-16.706	45.049	-17.278
116	ALA	O	-17.323	45.295	-18.363	116	ALA	CB	-18.811	45.518	-15.151
117	ASN	N	-15.423	45.398	-17.122	117	ASN	CA	-16.353	45.967	-18.139
117	ASN	C	-13.627	44.974	-19.034	117	ASN	O	-12.997	45.436	-19.820
117	ASN	CB	-13.615	46.958	-17.426	117	ASN	CG	-14.688	48.177	-16.939
117	ASN	OD1	-14.565	49.082	-17.773	117	ASN	ND2	-14.931	48.249	-15.736
118	ASN	N	-14.223	43.725	-18.967	118	ASN	CA	-13.760	42.642	-19.832
118	ASN	C	-12.240	42.444	-19.943	118	ASN	O	-11.617	42.309	-20.932
118	ASN	CB	-14.247	42.843	-21.279	118	ASN	CG	-15.737	43.060	-21.395
118	ASN	OD1	-16.310	42.321	-20.759	118	ASN	ND2	-16.136	44.096	-22.133
119	MET	N	-11.686	42.580	-18.675	119	MET	CA	-10.232	42.222	-18.478
119	MET	C	-10.025	40.734	-18.928	119	MET	O	-10.888	39.838	-18.759
119	MET	CB	-9.610	42.461	-17.055	119	MET	CG	-9.880	43.883	-16.982
119	MET	SD	-8.788	44.963	-17.526	119	MET	CE	-9.982	46.061	-18.263
120	ASP	N	-8.984	40.637	-19.584	120	ASP	CA	-8.488	39.118	-20.038
120	ASP	C	-7.822	38.398	-18.856	120	ASP	O	-8.038	37.189	-18.498
120	ASP	CB	-7.595	39.156	-21.236	120	ASP	CG	-8.237	39.738	-22.454
120	ASP	OD1	-7.881	40.786	-23.084	120	ASP	OD2	-9.327	39.135	-22.739
121	VAL	N	-7.071	39.117	-18.115	121	VAL	CA	-6.226	38.681	-16.976
121	VAL	C	-6.296	39.534	-15.796	121	VAL	O	-6.284	40.788	-15.989
121	VAL	CB	-4.735	38.587	-17.496	121	VAL	CG1	-5.798	38.176	-16.627
121	VAL	CG2	-4.707	37.916	-18.864	122	ILE	N	-6.318	38.978	-14.798
122	ILE	CA	-6.268	39.799	-13.597	122	ILE	C	-5.828	39.262	-12.627
122	ILE	O	-4.829	38.812	-12.669	122	ILE	CB	-7.476	39.684	-12.666
122	ILE	CG1	-8.686	40.392	-13.063	122	ILE	CG2	-7.221	39.883	-10.954
122	ILE	CD1	-9.976	39.788	-12.393	123	ASN	N	-6.263	40.222	-12.110
123	ASN	CA	-3.165	39.854	-11.232	123	ASN	C	-3.502	40.484	-9.861
123	ASN	O	-3.700	41.031	-9.833	123	ASN	CG	-1.028	40.478	-11.697
123	ASN	CG	-0.692	40.048	-10.777	123	ASN	OD1	-0.063	39.998	-11.018
123	ASN	ND2	-0.366	40.747	-9.720	124	MET	N	-3.458	39.604	-8.832
124	MET	CA	-3.458	39.973	-7.618	124	MET	C	-2.423	39.603	-8.614

124	MET	O	-2.306	30.888	-6.813	124	MET	CB	-4.943	38.587	-6.890
124	MET	CG	-6.198	40.002	-7.673	124	MET	SD	-7.883	38.472	-6.510
124	MET	CE	-7.960	38.991	-7.942	125	SER	N	-1.454	40.496	-6.802
125	SER	CA	-0.393	40.307	-3.769	125	SER	C	-0.422	40.712	-4.326
125	SER	O	0.238	41.617	-3.091	125	SER	CB	1.021	41.027	-4.320
125	SER	OG	1.444	40.696	-7.578	126	LEU	N	-1.433	40.878	-3.775
126	LEU	CA	-3.043	40.367	-2.306	126	LEU	C	-2.438	39.886	-1.887
126	LEU	O	-2.044	38.136	-2.879	126	LEU	CB	-2.791	41.868	-2.410
126	LEU	CG	-3.988	41.447	-3.333	126	LEU	CD1	-1.278	41.131	-2.578
126	LEU	CD2	-4.170	42.766	-4.073	127	GLY	N	-2.522	39.002	-0.481
127	GLY	CA	-3.038	37.871	0.193	127	GLY	C	-3.176	38.188	1.802
127	GLY	O	-2.444	39.832	2.225	128	GLY	N	-4.121	37.443	2.222
128	GLY	CA	-4.475	37.498	3.042	128	GLY	C	-4.644	36.038	4.186
128	GLY	O	-4.903	35.198	3.276	129	PRO	N	-4.519	35.857	5.402
129	PRO	CA	-4.671	34.323	5.998	129	PRO	C	-6.116	34.806	6.082
129	PRO	O	-6.338	32.887	6.303	129	PRO	CB	-4.840	34.484	7.384
129	PRO	CG	-4.439	30.316	7.727	129	PRO	CD	-4.239	36.076	6.618
130	SER	N	-7.051	33.013	5.912	130	SER	CA	-8.470	34.431	6.023
130	SER	C	-9.218	34.804	4.726	130	SER	O	-8.949	35.881	4.829
130	SER	CB	-9.049	35.351	7.216	130	SER	OG	-8.723	36.626	5.403
131	GLY	N	-10.083	33.967	4.349	131	GLY	CA	-10.826	34.229	3.074
131	GLY	C	-12.263	34.733	3.842	131	GLY	O	-12.495	34.722	4.781
132	SER	N	-13.940	33.038	2.594	132	SER	CA	-14.607	35.433	3.011
132	SER	C	-13.289	34.803	1.936	132	SER	O	-14.799	34.806	0.826
132	SER	CB	-14.590	30.927	3.145	132	SER	OG	-14.893	37.539	1.075
133	ALA	N	-16.547	34.588	2.204	133	ALA	CA	-17.807	34.857	1.326
133	ALA	C	-17.630	34.965	0.887	133	ALA	O	-17.763	34.437	-1.816
133	ALA	CB	-18.846	33.828	1.996	134	ALA	N	-17.683	36.288	0.296
134	ALA	CA	-17.872	37.289	-0.792	134	ALA	C	-16.635	37.369	-1.674
134	ALA	O	-16.781	37.585	-2.849	134	ALA	CB	-18.263	38.606	-0.187
135	LEU	N	-15.478	37.229	-3.046	135	LEU	CA	-14.197	37.244	-1.804
135	LEU	C	-14.198	36.905	-2.703	135	LEU	O	-13.796	36.820	-3.890
135	LEU	CB	-13.858	37.328	-0.798	135	LEU	CG	-11.893	37.130	-1.588
135	LEU	CD1	-11.460	38.415	-2.292	135	LEU	CD2	-10.582	36.857	-0.819
136	LYS	N	-16.809	34.823	-2.173	136	LYS	CA	-16.543	33.997	-3.013
136	LYS	C	-18.844	33.739	-4.190	136	LYS	C	-18.270	33.431	-5.303
136	LYS	CB	-14.903	32.341	-2.188	136	LYS	CG	-14.743	31.067	-3.043
136	LYS	CD	-13.083	29.892	-2.134	136	LYS	CE	-13.743	28.707	-2.776
136	LYS	NZ	-13.308	28.621	-4.160	137	ALA	N	-16.744	34.200	-3.867
137	ALA	CA	-17.793	34.416	-4.883	137	ALA	C	-17.338	35.303	-6.043
137	ALA	O	-17.708	33.049	-7.208	137	ALA	CB	-19.896	34.941	-6.283
138	ALA	N	-16.529	36.301	-5.729	138	ALA	CA	-16.881	37.311	-6.685
138	ALA	C	-16.903	36.696	-7.837	138	ALA	O	-14.985	36.843	-8.762
138	ALA	CB	-15.822	38.967	-5.934	139	VAL	N	-13.950	33.959	-7.027
139	VAL	CA	-12.946	33.291	-7.037	139	VAL	C	-13.623	34.228	-8.720
139	VAL	O	-13.208	34.070	-9.877	139	VAL	CB	-11.830	34.073	-6.960
139	VAL	CG1	-10.919	33.856	-7.866	139	VAL	CG2	-11.078	33.700	-6.333
140	ASP	N	-16.593	33.936	-8.322	140	ASP	CA	-15.276	32.496	-8.929
140	ASP	C	-16.023	33.131	-10.084	140	ASP	O	-16.880	32.879	-11.190
140	ASP	CB	-16.149	31.849	-9.198	140	ASP	CG	-15.388	30.448	-7.186
140	ASP	OD1	-16.178	30.483	-7.382	140	ASP	OD2	-16.339	30.132	-6.329
141	LYS	N	-16.038	34.203	-9.010	141	LYS	CA	-17.373	33.086	-10.868
141	LYS	C	-18.373	38.418	-11.946	141	LYS	O	-16.700	35.240	-13.111
141	LYS	CB	-18.039	36.278	-10.328	141	LYS	CG	-18.884	37.836	-11.306
141	LYS	CD	-19.806	38.187	-10.336	141	LYS	CE	-20.572	39.051	-11.230
141	LYS	NZ	-21.138	40.037	-10.878	142	ALA	N	-15.167	35.048	-11.866
142	ALA	CA	-14.173	36.192	-12.816	142	ALA	C	-13.818	38.810	-13.821
142	ALA	O	-13.770	35.169	-14.785	142	ALA	CB	-12.870	36.097	-11.940
143	VAL	N	-13.982	33.886	-12.832	143	VAL	CA	-13.160	32.703	-13.650
143	VAL	C	-14.346	32.235	-14.496	143	VAL	O	-14.140	31.084	-15.029
143	VAL	CB	-12.881	31.673	-12.716	143	VAL	CG1	-12.300	30.370	-13.461
143	VAL	CG2	-11.308	32.195	-12.816	144	ALA	N	-13.331	32.230	-13.075
144	ALA	CA	-16.744	33.834	-14.061	144	ALA	C	-16.928	32.681	-15.061

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144	ALA	C	-17.305	32.363	-16.953
145	SER	N	-16.337	33.948	-15.706
145	SER	C	-15.609	30.773	-17.829
145	SER	CB	-17.016	30.376	-16.616
146	GLY	N	-16.577	33.986	-17.565
146	GLY	C	-12.373	34.491	-18.385
147	VAL	N	-12.150	35.362	-17.304
147	VAL	C	-9.050	34.036	-16.323
147	VAL	CB	-11.152	36.977	-13.089
147	VAL	CG2	-12.360	37.915	-16.230
148	VAL	CA	-7.682	34.230	-15.008
148	VAL	O	-6.865	30.133	-14.750
148	VAL	CG1	-5.079	33.483	-16.361
149	VAL	N	-7.250	34.355	-13.331
149	VAL	C	-8.700	34.308	-11.633
149	VAL	CB	-6.224	36.090	-11.313
149	VAL	CG2	-9.456	35.386	-12.096
150	VAL	CA	-3.303	34.987	-10.901
150	VAL	O	-3.592	30.778	-9.400
150	VAL	CG1	-0.973	34.633	-11.461
151	ALA	N	-2.568	34.946	-8.595
151	ALA	C	-1.800	35.836	-6.657
151	ALA	CB	-3.557	35.390	-6.307
152	ALA	CA	0.714	33.630	-5.112
152	ALA	O	-0.728	34.466	-3.447
153	ALA	N	1.125	33.302	-3.012
153	ALA	C	0.931	32.725	-1.511
153	ALA	CB	1.750	31.030	-3.195
154	GLY	CA	2.043	34.221	0.125
154	GLY	O	4.189	33.267	-0.118
155	ASN	CA	5.344	34.787	2.037
155	ASN	O	6.101	34.829	4.295
155	ASN	CG	5.890	36.702	0.500
155	ASN	ND2	5.454	37.965	0.352
156	GLU	CA	4.633	32.537	4.970
156	GLU	O	5.376	30.637	6.222
156	GLU	CG	2.491	32.442	6.360
156	GLU	OE1	1.764	34.322	5.312
157	GLY	N	6.309	31.057	4.227
157	GLY	C	6.503	28.622	4.553
158	THR	N	7.147	27.793	5.382
158	THR	OG1	8.707	25.487	6.217
158	THR	CA	6.552	26.487	8.782
158	THR	O	8.479	27.335	7.977
159	SER	OG	3.141	25.904	10.525
159	SER	CA	4.031	25.210	8.055
159	SER	O	3.339	23.281	9.030
160	GLY	CA	5.636	21.904	8.095
160	GLY	O	4.800	21.326	6.555
161	SER	CA	2.616	19.777	7.056
161	SER	O	0.696	20.347	5.849
161	SER	OG	1.034	18.029	6.555
162	SER	CA	0.167	22.725	7.113
162	SER	O	1.333	23.840	5.396
162	SER	OG	8.304	23.891	9.460
163	SER	CA	-0.611	24.750	3.990
163	SER	O	-1.070	26.560	5.504
163	SER	OG	-1.092	25.710	2.331
164	THR	CA	0.609	28.340	4.311
164	THR	O	5.465	30.582	5.278
164	THR	OG1	2.984	28.382	3.692
165	VAL	N	-0.515	28.742	2.190
165	VAL	C	-2.018	30.543	1.497
144	ALA	CB	-17.962	31.960	-13.708
145	SER	CA	-16.682	34.017	-16.706
145	SER	O	-15.918	35.321	-18.573
145	SER	OG	-15.882	36.915	-15.949
146	GLY	CA	-13.619	33.709	-18.673
146	GLY	O	-11.420	34.306	-19.266
147	VAL	CA	-10.874	35.836	-16.913
147	VAL	O	-10.371	33.991	-15.486
147	VAL	CG1	-9.896	37.003	-15.578
148	VAL	N	-8.983	35.018	-16.603
148	VAL	C	-7.157	34.907	-14.701
148	VAL	CB	-6.273	34.126	-16.950
148	VAL	CG2	-6.196	33.432	-18.262
149	VAL	CA	-6.987	34.965	-13.249
149	VAL	O	-5.626	33.173	-11.639
149	VAL	CG1	-7.893	35.619	-10.089
150	VAL	N	-4.722	35.301	-11.606
150	VAL	C	-3.157	35.625	-9.559
150	VAL	CB	-2.276	35.305	-11.951
150	VAL	CG2	-2.678	34.863	-13.301
151	ALA	CA	-2.341	35.882	-7.287
151	ALA	O	-0.618	33.889	-6.906
152	ALA	N	-0.690	35.907	-5.012
152	ALA	C	0.304	34.320	-6.138
152	ALA	CB	1.366	36.607	-4.296
153	ALA	CA	0.848	32.258	-2.963
153	ALA	O	0.317	32.192	-0.899
154	GLY	N	1.827	33.693	-1.266
154	GLY	C	3.519	34.069	0.558
155	ASN	N	3.958	34.708	1.960
155	ASN	C	5.390	34.258	3.662
155	ASN	CB	6.088	36.190	2.906
155	ASN	OD1	6.123	34.063	-0.536
156	GLU	N	4.711	33.168	3.675
156	GLU	C	5.322	31.320	5.303
156	GLU	CB	3.205	31.900	5.100
156	GLU	CD	2.304	33.951	6.270
156	GLU	OE2	3.106	34.036	7.166
157	GLY	CA	7.306	29.917	4.987
157	GLY	O	5.616	28.346	4.009
158	THR	CG2	8.979	25.396	3.859
158	THR	CB	7.564	25.344	5.296
158	THR	C	6.150	26.409	7.157
159	SER	N	5.338	25.441	7.497
159	SER	CB	3.673	26.105	9.212
159	SER	C	4.494	23.720	8.964
160	GLY	N	5.576	22.967	8.835
160	GLY	C	4.576	21.049	7.730
161	SER	N	3.925	20.310	8.316
161	SER	C	1.677	20.780	6.706
161	SER	CB	2.366	18.293	7.271
162	SER	N	1.303	21.041	7.699
162	SER	C	0.630	23.552	5.868
162	SER	CB	-0.213	23.666	5.362
163	SER	N	-0.679	23.921	5.197
163	SER	C	-0.441	26.177	4.313
163	SER	CB	-1.890	26.662	3.231
164	THR	N	0.387	28.932	3.032
164	THR	C	0.183	29.286	3.194
164	THR	CB	2.099	28.510	4.810
164	THR	CG2	2.397	27.610	6.001
165	VAL	CA	-0.959	29.562	1.010
165	VAL	O	-2.929	30.152	2.200

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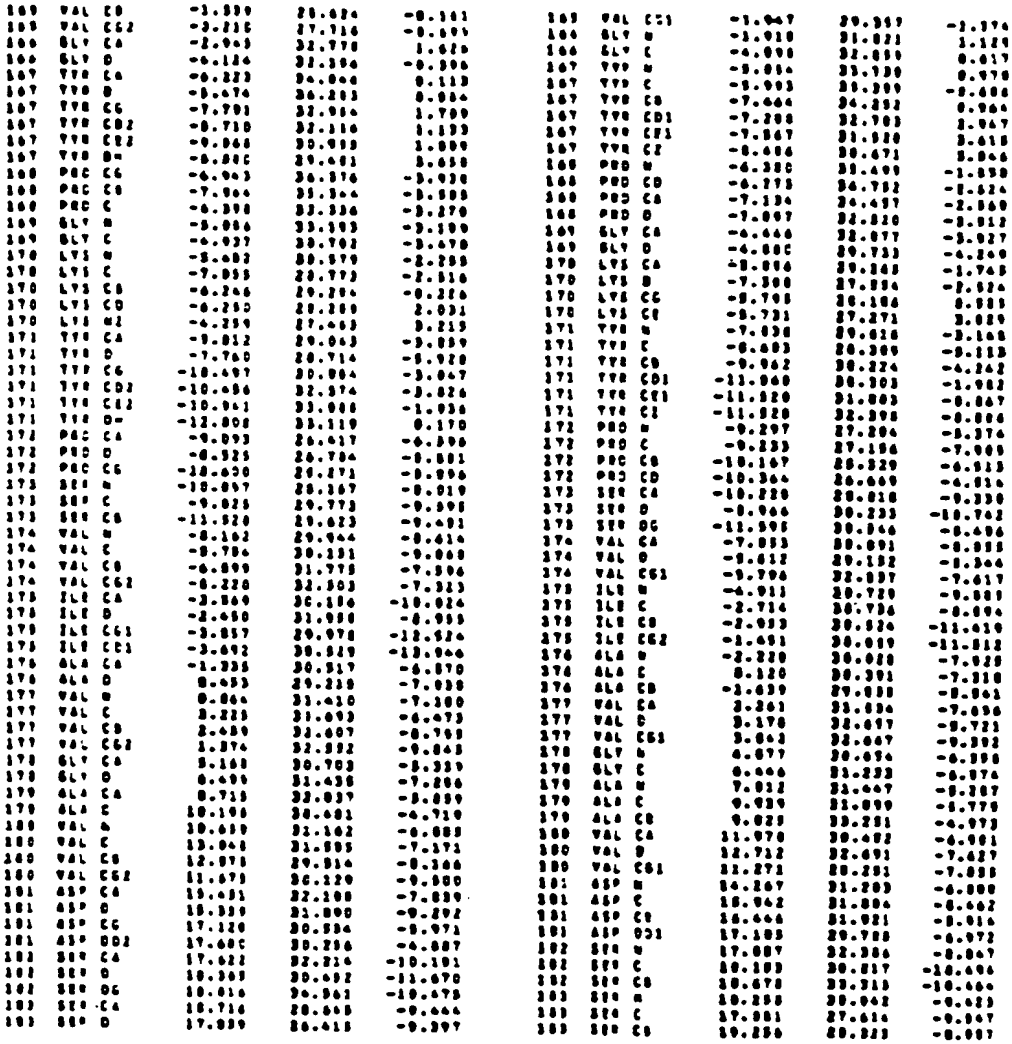

183	SER OG	20.889	28.615	-8.231	184	ASN N	18.373	28.896	-9.882
184	ASN CA	13.144	27.317	-8.980	184	ASN C	14.931	26.720	-8.197
184	ASN O	14.130	25.789	-8.897	184	ASN CB	15.816	26.361	-10.722
184	ASN CG	14.993	26.998	-12.070	184	ASN OD1	16.789	28.106	-13.277
184	ASN ND2	15.352	26.210	-13.070	185	GLN N	15.862	27.267	-7.188
185	GLN CA	15.270	26.046	-5.833	185	GLN C	14.280	27.694	-5.283
185	GLN O	14.189	28.726	-5.386	185	GLN CB	16.899	26.568	-5.101
185	GLN CG	16.539	26.242	-3.616	185	GLN CD	18.011	26.102	-3.286
185	GLN OE1	18.866	25.799	-4.081	185	GLN NE2	18.266	26.386	-1.936
186	ARG N	13.270	26.938	-4.448	186	ARG CA	12.185	27.776	-3.841
186	ARG C	12.700	28.782	-2.866	186	ARG O	13.698	28.304	-2.093
186	ARG CB	11.215	26.943	-3.116	186	ARG CG	10.216	27.471	-2.161
186	ARG CD	9.467	26.337	-1.481	186	ARG NE	9.866	26.333	-0.117
186	ARG CZ	9.961	26.879	1.839	186	ARG NH1	9.367	27.888	1.688
186	ARG NH2	10.966	26.321	1.783	187	ALA N	12.296	30.889	-2.883
187	ALA CA	12.720	31.064	-1.893	187	ALA C	12.262	30.686	-0.817
187	ALA O	11.158	30.863	-0.387	187	ALA CB	12.166	32.682	-2.366
188	SER N	13.891	32.770	0.549	188	SER CA	12.671	30.886	1.868
188	SER C	11.336	30.867	2.412	188	SER O	10.740	30.111	3.212
188	SER CB	13.767	30.456	2.938	188	SER OG	14.137	31.826	2.841
189	PHE N	10.963	32.010	1.974	189	PHE CA	9.697	32.688	2.418
189	PHE C	8.499	32.198	1.609	189	PHE O	7.389	32.886	2.011
189	PHE CB	9.787	34.217	2.243	189	PHE CG	10.117	34.696	0.867
189	PHE CD1	9.387	34.830	-0.131	189	PHE CD2	11.415	35.116	0.967
189	PHE CE1	9.683	33.187	-1.411	189	PHE CE2	11.769	35.863	-0.701
189	PHE CZ	10.786	35.886	-1.728	190	SER N	8.783	31.826	0.699
190	SER CA	7.626	31.896	-0.391	190	SER C	6.663	30.162	0.328
190	SER O	7.836	29.883	0.866	190	SER CB	8.181	30.890	-1.788
190	SER OG	7.136	30.337	-2.618	191	SER N	5.388	30.951	0.326
191	SER CA	6.361	29.676	0.987	191	SER C	4.261	28.330	0.223
191	SER O	6.863	28.268	-0.895	191	SER CB	3.838	30.611	0.911
191	SER OG	2.729	33.285	1.936	192	VAL N	3.786	27.310	0.920
192	VAL CA	3.627	28.932	0.391	192	VAL C	2.286	28.291	0.886
192	VAL O	1.899	28.698	1.198	192	VAL CB	4.781	28.127	1.888
192	VAL CG1	0.164	28.727	0.722	192	VAL CG2	4.617	29.306	2.892
193	GLY N	1.938	24.172	0.067	193	GLY CA	0.629	23.566	0.618
193	GLY C	0.081	23.029	-0.901	193	GLY O	0.530	23.266	-2.819
194	PRO N	-1.023	22.281	-0.722	194	PRO CA	-1.662	21.651	-1.873
194	PRO C	-2.237	22.685	-2.916	194	PRO O	-2.603	22.266	-4.808
194	PRO CB	-2.769	20.783	-1.210	194	PRO CG	-2.311	20.632	0.213
194	PRO CD	-1.633	21.986	0.570	195	GLU N	-2.822	23.793	-2.638
195	GLU CA	-3.145	24.830	-3.252	195	GLU C	-2.893	25.631	-4.858
195	GLU O	-2.916	26.398	-4.936	195	GLU CB	-4.063	28.788	-2.670
195	GLU CG	-4.962	28.136	-1.635	195	GLU CD	-4.318	26.860	-0.188
195	GLU OE1	-3.110	26.960	0.165	195	GLU OE2	-5.138	26.820	0.783
196	LEU N	-0.829	25.264	-3.070	196	LEU CA	0.241	28.929	-4.666
196	LEU C	0.328	25.376	-6.059	196	LEU O	0.305	26.122	-6.183
196	LEU CB	1.340	25.789	-3.884	196	LEU CG	2.770	28.170	-4.663
196	LEU CD1	2.739	27.716	-6.631	196	LEU CD2	4.027	25.721	-3.911
197	ASP N	0.140	26.208	-7.093	197	ASP CA	0.932	25.776	-8.488
197	ASP C	1.387	28.731	-9.293	197	ASP O	1.033	26.734	-9.016
197	ASP CB	-1.067	26.598	-9.191	197	ASP CG	-2.606	26.351	-8.548
197	ASP OD1	-2.886	28.155	-8.376	197	ASP OD2	-3.035	27.317	-8.008
198	VAL N	2.033	26.089	-9.344	198	VAL CA	3.206	26.970	-10.299
198	VAL C	4.187	27.950	-9.316	198	VAL O	3.782	28.699	-8.587
198	VAL CB	2.896	27.476	-11.637	198	VAL CG1	1.930	26.726	-12.937
198	VAL CG2	2.337	28.919	-12.686	199	MET N	5.376	27.916	-10.016
199	MET CA	6.439	28.807	-9.498	199	MET C	6.065	29.019	-10.878
199	MET O	6.696	29.318	-11.793	199	MET CB	7.660	27.070	-9.077
199	MET CG	7.361	26.869	-8.139	199	MET SD	6.783	27.469	-6.868
199	MET CE	8.227	27.789	-5.387	200	ALA N	7.626	30.942	-10.183
200	ALA CA	7.991	31.029	-11.088	200	ALA C	9.888	32.666	-10.272
200	ALA O	8.127	32.814	-9.060	200	ALA CB	6.932	32.070	-11.638

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201	PRO	N	9.927	35.455	-10.993	201	PRO	CA	11.013	34.110	-10.230
201	PRO	C	10.458	35.127	-9.238	201	PRO	O	9.579	35.987	-9.482
201	PRO	CB	11.027	34.733	-11.400	201	PRO	CG	11.393	36.040	-12.570
201	PRO	CD	9.961	33.616	-12.405	202	GLY	N	10.921	35.204	-8.021
202	GLY	CA	10.473	36.234	-7.044	202	GLY	C	11.309	36.658	-6.111
202	GLY	O	11.312	37.126	-6.979	203	VAL	N	12.835	36.503	-6.613
203	VAL	CA	13.948	36.919	-5.716	203	VAL	C	14.706	38.017	-6.469
203	VAL	C	13.133	37.731	-7.593	203	VAL	CB	16.814	35.600	-5.381
203	VAL	CG1	16.096	36.106	-4.612	203	VAL	CG2	14.879	36.763	-4.370
204	SER	N	14.185	39.102	-5.059	204	SER	CA	15.872	40.281	-6.487
204	SER	C	15.067	40.619	-7.072	204	SER	C	15.706	40.683	-8.889
204	SER	CB	17.087	39.976	-6.326	204	SER	OG	17.732	41.106	-6.472
205	ILE	N	13.771	42.965	-8.008	205	ILE	CA	13.069	41.234	-9.225
205	ILE	C	13.207	42.740	-9.678	205	ILE	O	13.675	43.498	-8.640
205	ILE	CB	11.132	40.833	-9.144	205	ILE	CG1	11.436	39.336	-8.810
205	ILE	CG2	10.599	41.283	-10.467	205	ILE	CD1	12.257	38.412	-9.771
206	GLN	N	13.086	43.395	-10.409	206	GLN	CA	16.206	44.317	-10.834
206	GLN	C	13.102	44.978	-11.630	206	GLN	O	12.669	44.318	-12.621
206	GLN	CB	15.485	44.708	-11.740	206	GLN	CG	16.686	44.163	-10.989
206	GLN	CD	17.285	45.165	-10.887	206	GLN	OE1	18.328	44.936	-9.333
206	GLN	NE2	16.536	46.260	-9.857	207	SER	N	12.359	46.866	-11.214
207	SER	CA	11.217	46.573	-11.987	207	SER	C	11.089	46.893	-11.749
207	SER	O	11.919	45.657	-11.006	207	SER	CB	9.918	45.833	-11.869
207	SER	OG	8.993	46.856	-12.613	208	THR	N	10.854	48.666	-13.326
208	THR	CG2	9.171	50.339	-14.796	208	THR	OG1	7.570	49.414	-13.166
208	THR	CB	8.420	50.415	-13.397	208	THR	CA	9.675	50.092	-13.173
208	THR	C	9.197	50.488	-10.803	208	THR	O	8.423	49.887	-10.049
209	LEU	N	9.676	51.613	-10.220	209	LEU	CA	9.192	52.151	-8.959
209	LEU	C	8.873	53.610	-9.262	209	LEU	O	9.140	54.227	-10.222
209	LEU	CB	10.335	52.392	-7.938	209	LEU	CG	10.806	53.816	-7.616
209	LEU	CD1	11.968	51.116	-6.472	209	LEU	CD2	9.687	50.282	-6.649
210	PRO	N	7.790	54.339	-6.444	210	PRO	CA	7.273	55.017	-8.069
210	PRO	C	8.383	56.573	-6.639	210	PRO	O	9.401	56.668	-8.106
210	PRO	CB	6.302	55.733	-7.917	210	PRO	CG	6.006	56.370	-6.966
210	PRO	CD	7.103	53.493	-7.271	211	GLY	N	8.077	57.661	-9.351
211	GLY	CA	9.069	58.763	-9.610	211	GLY	C	10.096	58.636	-10.490
211	GLY	O	11.176	59.008	-10.289	212	ASN	N	9.851	57.770	-11.087
212	ASN	CA	10.903	57.422	-11.643	212	ASN	C	12.039	56.793	-12.056
212	ASN	C	13.108	57.361	-12.620	212	ASN	CB	11.226	58.393	-13.499
212	ASN	CG	11.803	58.109	-14.834	212	ASN	OD1	11.853	57.856	-15.323
212	ASN	ND2	12.273	59.159	-15.376	213	LYS	N	11.803	55.769	-11.247
213	LYS	CA	12.810	54.946	-10.337	213	LYS	C	12.668	53.687	-10.966
213	LYS	O	11.773	53.039	-11.613	213	LYS	CB	12.769	55.261	-8.859
213	LYS	CG	13.206	56.696	-8.767	213	LYS	CD	13.246	57.080	-7.312
213	LYS	CE	14.195	58.218	-6.870	213	LYS	NZ	15.068	58.705	-7.921
214	TYR	N	13.681	52.783	-10.444	214	TYR	CA	13.803	51.346	-10.722
214	TYR	C	14.383	50.650	-9.489	214	TYR	O	15.211	51.393	-8.817
214	TYR	CB	16.461	50.981	-11.984	214	TYR	CG	14.330	51.621	-13.246
214	TYR	CD1	14.689	51.047	-13.678	214	TYR	CD2	13.129	51.063	-14.016
214	TYR	CE1	16.230	53.478	-14.814	214	TYR	CE2	12.656	51.669	-15.178
214	TYR	CZ	13.204	52.895	-15.850	214	TYR	OH	12.786	53.458	-16.496
215	GLY	N	16.850	49.867	-9.158	215	GLY	CA	16.622	48.772	-7.005
215	GLY	C	16.130	47.320	-7.969	215	GLY	O	15.269	46.917	-8.521
216	ALA	N	16.810	46.680	-8.031	216	ALA	CA	16.656	45.203	-6.701
216	ALA	C	15.602	44.922	-8.312	216	ALA	O	15.048	45.527	-4.678
216	ALA	CB	15.731	44.356	-8.887	217	TYR	N	12.788	43.982	-5.578
217	TYR	CA	11.964	43.488	-4.446	217	TYR	C	12.033	43.928	-4.567
217	TYR	O	12.262	41.442	-5.656	217	TYR	CB	10.673	43.862	-4.570
217	TYR	CG	10.117	45.293	-4.216	217	TYR	CD1	10.846	45.991	-3.136
217	TYR	CD2	9.016	45.933	-4.709	217	TYR	CE1	10.489	47.207	-2.790
217	TYR	CE2	8.656	47.210	-4.301	217	TYR	CZ	9.358	47.882	-3.302
217	TYR	OH	8.933	48.160	-2.980	218	ASN	N	11.790	41.306	-3.391
218	ASN	CA	11.046	39.942	-3.227	218	ASN	C	10.204	39.036	-2.760

218	ASN	O	9.763	43.347	-1.017	218	ASN	CB	12.913	39.340	-3.184
218	ASN	CG	14.031	39.566	-2.343	218	ASN	OD1	14.612	39.788	-3.422
218	ASN	ND2	14.668	39.664	-1.161	219	GLY	N	9.478	38.934	-3.209
219	GLY	CA	8.382	38.135	-2.649	219	GLY	C	7.078	37.304	-3.681
219	GLY	O	7.873	37.801	-4.876	220	THR	N	6.161	36.638	-3.205
220	THR	CA	5.697	35.936	-4.179	220	THR	C	4.879	37.044	-4.864
220	THR	O	4.617	36.762	-5.018	220	THR	CB	4.825	34.819	-3.926
220	THR	OG1	4.136	35.543	-2.691	220	THR	CG2	5.784	33.696	-2.988
221	SER	N	4.738	38.238	-4.363	221	SER	CA	3.984	39.261	-5.169
221	SER	C	4.760	39.641	-6.385	221	SER	O	4.117	40.208	-7.277
221	SER	CB	3.319	40.283	-4.546	221	SER	OG	3.439	40.262	-3.144
222	MET	N	5.843	39.389	-6.485	222	MET	CE	8.671	42.771	-8.171
222	MET	SD	7.768	41.533	-4.993	222	MET	CG	8.866	41.399	-6.602
222	MET	CB	6.351	40.818	-7.218	222	MET	CA	6.916	39.678	-7.638
222	MET	C	6.877	38.435	-8.567	222	MET	O	7.884	38.867	-9.778
223	ALA	N	6.896	37.246	-8.041	223	ALA	CA	6.469	36.828	-8.885
223	ALA	C	5.200	36.068	-9.707	223	ALA	O	5.133	35.940	-10.929
223	ALA	CB	8.305	34.807	-7.923	224	SER	N	4.878	36.369	-9.838
224	SER	CA	3.758	36.488	-8.703	224	SER	C	2.661	37.161	-11.839
224	SER	O	2.145	36.693	-12.837	224	SER	CB	1.801	36.995	-9.483
224	SER	OG	6.492	36.899	-9.197	225	PRO	N	3.156	38.411	-11.199
225	PRO	CA	3.095	39.130	-12.439	225	PRO	C	3.764	38.469	-13.624
225	PRO	O	3.406	38.690	-14.804	225	PRO	CB	3.653	40.511	-12.884
225	PRO	CG	4.611	40.402	-10.764	225	PRO	CD	3.735	39.224	-10.884
226	HIS	N	4.769	37.624	-13.299	226	HIS	CA	5.446	36.879	-14.862
226	HIS	C	4.418	35.947	-15.861	226	HIS	O	4.425	35.859	-16.293
226	HIS	CB	6.688	36.846	-13.765	226	HIS	CG	7.814	36.859	-13.358
226	HIS	ND1	8.648	37.488	-12.170	226	HIS	CD2	8.883	37.118	-14.167
226	HIS	CE1	9.178	36.852	-12.236	226	HIS	NE2	9.771	37.866	-13.443
227	VAL	N	3.593	35.366	-14.199	227	VAL	CA	2.883	34.388	-14.721
227	VAL	C	1.679	33.197	-15.621	227	VAL	O	1.016	34.773	-14.496
227	VAL	CB	2.383	33.444	-13.439	227	VAL	CG1	1.076	32.676	-14.266
227	VAL	CG2	3.204	32.665	-12.891	228	ALA	N	1.803	36.262	-14.814
228	ALA	CA	0.811	37.189	-15.917	228	ALA	C	0.843	37.938	-16.868
228	ALA	O	-0.193	37.488	-17.820	228	ALA	CB	-0.307	38.353	-14.648
229	GLY	N	1.791	38.828	-16.961	229	GLY	CA	2.392	38.408	-18.229
229	GLY	C	2.428	37.197	-19.187	229	GLY	O	2.109	37.375	-20.384
230	ALA	N	2.711	35.986	-18.646	230	ALA	CA	2.796	34.801	-19.964
230	ALA	C	1.424	34.800	-20.183	230	ALA	O	1.380	34.265	-21.363
230	ALA	CB	3.298	33.624	-18.789	231	ALA	N	0.395	34.623	-19.328
231	ALA	CA	-1.010	34.616	-19.744	231	ALA	C	-1.286	33.429	-20.864
231	ALA	O	-1.989	35.054	-21.892	231	ALA	CB	-1.932	34.664	-18.849
232	ALA	N	-0.778	36.637	-21.721	232	ALA	CA	-1.813	37.663	-21.792
232	ALA	C	-0.281	37.204	-23.078	232	ALA	O	-0.841	37.801	-24.187
232	ALA	CB	-0.742	39.121	-21.377	233	LEU	N	0.938	36.726	-22.967
233	LEU	CA	1.617	36.293	-24.209	233	LEU	C	0.821	35.169	-24.886
233	LEU	O	0.456	35.231	-26.111	233	LEU	CB	3.043	35.877	-23.987
233	LEU	CG	3.996	36.994	-23.453	233	LEU	CD1	5.239	36.362	-22.921
233	LEU	CD2	4.241	37.833	-24.686	234	ILE	N	0.337	34.199	-24.067
234	ILE	CD1	0.306	38.664	-21.657	234	ILE	CG1	0.496	31.223	-23.188
234	ILE	CB	-0.811	32.914	-23.570	234	ILE	CG2	-1.003	36.980	-24.091
234	ILE	CA	-0.496	33.876	-24.644	234	ILE	C	-1.021	33.997	-25.496
235	ILE	O	-1.883	33.144	-26.544	235	LEU	N	-1.196	34.443	-24.778
235	LEU	CA	-3.306	35.018	-25.623	235	LEU	C	-3.258	33.843	-26.871
235	LEU	O	-4.109	35.814	-27.584	235	LEU	CE	-4.432	33.769	-24.978
235	LEU	CG	-5.140	34.899	-23.262	235	LEU	CD1	-5.652	33.603	-22.149
235	LEU	CD2	-6.152	34.138	-24.120	236	SER	N	-2.894	36.438	-26.798
236	SER	CA	-1.764	37.237	-27.986	236	SER	C	-1.491	36.292	-29.144
236	SER	O	-1.744	36.634	-30.296	236	SER	CB	-0.633	38.234	-27.733
236	SER	OG	0.598	37.571	-27.582	237	LYS	N	-1.046	35.867	-28.082
237	LYS	CA	-0.868	36.085	-29.952	237	LYS	C	-2.113	33.277	-30.269
237	LYS	O	-2.178	32.951	-31.444	237	LYS	CE	0.172	33.112	-29.991
237	LYS	CG	0.677	34.240	-30.716	237	LYS	CD	2.020	31.938	-30.442

237	LYS	CE	2.341	30.762	-31.720
238	HIS	N	-2.931	31.000	-30.312
238	HIS	C	-5.336	32.009	-28.697
238	HIS	CB	-3.948	30.082	-28.831
238	HIS	ND1	-3.707	29.470	-28.838
238	HIS	CE1	-3.006	28.051	-29.642
239	PRO	N	-3.048	33.927	-29.365
239	PRO	C	-8.204	34.252	-28.337
239	PRO	CB	-7.010	35.977	-29.713
239	PRO	CD	-5.436	34.430	-30.600
240	ASN	CA	-9.320	32.041	-29.216
240	ASN	O	-10.348	30.610	-27.176
240	ASN	CG	-7.071	30.827	-30.089
240	ASN	ND2	-7.676	29.300	-30.996
241	TRP	CA	-8.304	30.124	-26.126
241	TRP	O	-9.063	31.033	-26.404
241	TRP	CG	-6.896	28.903	-26.517
241	TRP	CD2	-6.039	28.326	-26.195
241	TRP	CE2	-6.616	27.476	-27.216
241	TRP	CZ2	-3.395	26.786	-27.176
241	TRP	CH2	-2.670	26.873	-26.085
242	THR	CA	-10.458	30.119	-22.933
242	THR	O	-8.335	29.476	-23.937
242	THR	OG1	-10.037	27.786	-22.476
243	ASN	N	-9.046	30.459	-20.611
243	ASN	OD1	-11.465	31.518	-16.760
243	ASN	CB	-9.788	31.030	-18.332
243	ASN	C	-8.657	29.363	-19.010
244	THR	N	-9.366	28.362	-19.283
244	THR	C	-8.133	26.393	-19.802
244	THR	CB	-10.665	26.888	-18.406
244	THR	CG2	-10.533	24.595	-19.199
245	GLN	CA	-6.964	26.342	-21.862
245	GLN	O	-4.373	26.393	-21.467
245	GLN	CG	-8.268	25.526	-23.989
245	GLN	OE1	-9.306	26.769	-25.727
246	VAL	N	-5.697	28.306	-21.210
246	VAL	C	-3.036	28.442	-19.467
246	VAL	CB	-4.779	30.555	-20.621
246	VAL	CG2	-5.169	31.330	-21.959
247	ARG	CA	-4.385	27.736	-17.168
247	ARG	O	-2.708	25.905	-16.764
247	ARG	CG	-4.987	27.898	-14.832
247	ARG	NE	-8.448	26.787	-12.546
247	ARG	NH1	-7.064	27.484	-11.319
248	SER	N	-4.480	25.509	-10.331
248	SER	C	-2.657	24.096	-10.072
248	SER	CB	-5.036	23.400	-19.372
249	SER	N	-2.300	24.083	-20.136
249	SER	C	-6.071	23.301	-19.948
249	SER	CB	-1.369	23.758	-22.088
250	LEU	N	-0.289	26.333	-19.360
250	LEU	CD1	-0.373	28.433	-17.268
250	LEU	CG	0.178	28.083	-17.803
250	LEU	C	1.092	28.696	-17.283
251	GLN	N	0.068	25.827	-16.716
251	GLN	OE1	-2.819	23.626	-12.938
251	GLN	CG	-1.218	24.816	-13.996
251	GLN	CA	0.381	23.941	-13.768
251	GLN	O	1.743	22.816	-15.618
252	ASN	CA	1.302	21.786	-18.202
252	ASN	O	2.099	20.462	-19.768
252	ASN	CG	-1.036	19.926	-18.373
237	LYS	NZ	0.020	29.848	-31.996
238	HIS	CA	-4.100	32.163	-29.179
238	HIS	O	-5.713	32.806	-27.563
238	HIS	CG	-3.009	29.021	-29.237
238	HIS	CD2	-3.337	29.298	-30.396
238	HIS	NE2	-1.968	28.688	-30.309
239	PRO	CA	-6.900	34.770	-28.773
239	PRO	O	-8.949	34.819	-27.663
239	PRO	CG	-6.646	33.296	-31.037
240	ASN	N	-8.386	32.069	-30.227
240	ASN	C	-9.500	31.100	-27.980
240	ASN	CB	-9.493	31.269	-30.036
240	ASN	OD1	-7.008	31.900	-31.147
241	TRP	N	-8.316	31.006	-27.304
241	TRP	C	-9.106	30.636	-24.936
241	TRP	CB	-6.079	29.036	-25.670
241	TRP	CD1	-6.330	28.433	-27.030
241	TRP	NE1	-5.362	27.367	-28.211
241	TRP	CE3	-4.097	28.406	-26.901
241	TRP	CZ3	-2.912	27.667	-26.063
242	THR	N	-9.727	29.781	-24.362
242	THR	C	-9.669	30.176	-21.767
242	THR	CB	-11.079	29.032	-22.675
242	THR	CG2	-12.406	28.907	-23.000
243	ASN	ND2	-11.787	30.686	-18.767
243	ASN	CG	-11.003	31.331	-17.908
243	ASN	CA	-9.033	30.731	-19.666
243	ASN	O	-7.893	29.136	-18.660
244	THR	CA	-9.381	26.934	-19.839
244	THR	O	-7.326	25.797	-19.111
244	THR	OG1	-11.730	26.675	-18.686
245	GLN	N	-8.682	26.716	-21.073
245	GLN	C	-5.647	27.020	-21.520
245	GLN	CB	-7.330	26.099	-23.397
245	GLN	CD	-8.493	25.073	-25.628
245	GLN	NE2	-7.765	25.312	-26.370
246	VAL	CA	-4.477	29.040	-20.770
246	VAL	O	-2.789	28.227	-19.361
246	VAL	CG1	-3.946	31.272	-20.027
247	ARG	N	-4.767	28.260	-18.462
247	ARG	C	-3.770	26.292	-17.360
247	ARG	CB	-3.833	27.467	-16.169
247	ARG	CD	-4.836	27.179	-13.793
247	ARG	CZ	-8.093	26.066	-11.318
247	ARG	NH2	-8.177	26.428	-10.270
248	SER	CA	-6.039	24.131	-18.626
248	SER	O	-1.868	23.293	-18.083
248	SER	OG	-6.166	23.098	-18.032
249	SER	CA	-1.223	24.574	-20.031
249	SER	O	1.026	24.781	-20.069
249	SER	OG	-0.300	25.619	-22.956
250	LEU	CD2	1.026	29.016	-18.222
250	LEU	CB	0.332	29.438	-18.181
250	LEU	CA	0.718	28.037	-18.216
250	LEU	C	1.393	25.421	-17.032
251	GLN	NE2	-2.780	25.312	-12.237
251	GLN	CD	-2.348	24.550	-13.036
251	GLN	CB	-0.557	23.621	-14.077
251	GLN	C	0.959	22.444	-16.301
252	ASN	N	0.633	22.306	-17.306
252	ASN	C	2.394	21.309	-18.991
252	ASN	CB	0.004	20.780	-19.292
252	ASN	OD1	-0.036	19.388	-17.582

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252	ASN ND2	-2.234	19.034	-19.161	253	THR N	5.819	22.885	-18.923
253	THR CA	4.230	22.717	-19.713	253	THR C	5.301	23.247	-18.811
253	THR O	6.340	25.733	-19.627	253	THR CB	4.991	23.672	-21.452
253	THR OG1	3.991	24.037	-20.628	253	THR CG2	3.147	23.138	-22.032
254	THR N	5.218	23.177	-17.551	254	THR CA	6.216	23.612	-16.588
254	THR C	7.466	22.730	-16.612	254	THR O	7.402	21.980	-17.095
254	THR CB	5.664	23.858	-15.132	254	THR OG1	5.121	22.178	-15.048
254	THR CG2	4.533	24.549	-14.882	255	THR N	8.699	23.396	-16.076
255	THR CA	9.771	22.596	-15.017	255	THR C	9.621	22.031	-14.414
255	THR O	9.430	22.786	-13.674	255	THR CB	11.083	23.451	-16.897
255	THR OG1	11.082	23.709	-17.321	255	THR CG2	12.286	22.628	-15.606
256	LYS N	9.696	20.702	-14.314	256	LYS CA	9.366	20.063	-13.010
256	LYS C	10.512	20.333	-12.063	256	LYS O	11.082	20.276	-12.892
256	LYS CB	9.026	18.590	-13.269	256	LYS CG	9.818	17.805	-11.921
256	LYS CD	10.216	16.948	-11.777	256	LYS CE	10.212	15.960	-10.613
256	LYS NZ	9.263	14.969	-11.056	257	LEU N	10.212	20.674	-10.626
257	LEU CA	11.272	21.036	-9.693	257	LEU C	11.215	20.232	-8.616
257	LEU O	12.096	20.343	-7.732	257	LEU CB	11.187	22.547	-9.822
257	LEU CG	11.397	23.620	-10.368	257	LEU CD1	11.248	25.003	-9.921
257	LEU CD2	12.678	23.468	-11.325	258	GLY N	10.631	19.282	-8.218
258	GLY CA	10.062	18.793	-6.079	258	GLY C	9.160	18.703	-6.373
258	GLY O	8.283	18.956	-7.262	259	ASP N	9.826	18.282	-5.150
259	ASP CA	7.757	17.696	-6.316	259	ASP C	6.659	18.961	-4.709
259	ASP O	6.859	20.039	-6.216	259	ASP CB	7.996	17.960	-3.853
259	ASP CG	6.701	17.128	-2.261	259	ASP OD1	5.611	17.927	-2.356
259	ASP OD2	7.898	16.209	-1.321	260	SER N	5.563	18.610	-5.312
260	SER CA	4.481	19.597	-3.529	260	SER C	4.046	20.342	-4.289
260	SER C	3.306	21.563	-4.446	260	SER CB	3.345	18.919	-6.289
260	SER OG	2.748	17.937	-5.448	261	PHE N	4.241	19.778	-3.112
261	PHE CA	3.033	20.460	-1.885	261	PHE C	4.544	21.846	-1.063
261	PHE O	3.944	22.040	-1.432	261	PHE CB	4.053	19.769	-0.863
261	PHE CG	3.549	20.337	0.719	261	PHE CD1	2.286	20.163	1.123
261	PHE CD2	4.491	21.066	1.898	261	PHE CE1	1.737	20.717	2.318
261	PHE CE2	3.963	21.662	2.748	261	PHE CZ	2.605	21.463	3.116
262	TYR N	5.778	21.788	-2.303	262	TYR CA	6.608	22.914	-3.231
262	TYR C	6.826	23.689	-3.945	262	TYR O	7.201	24.553	-3.393
262	TYR CB	8.123	22.433	-1.851	262	TYR CG	8.146	21.302	-0.656
262	TYR CD1	8.084	20.484	-0.364	262	TYR CD2	8.149	22.661	0.698
262	TYR CE1	8.862	19.873	0.882	262	TYR CE2	8.116	22.069	1.962
262	TYR CZ	8.869	20.672	2.918	262	TYR OH	7.965	20.029	3.209
263	TYR N	6.626	23.104	-6.693	263	TYR CA	6.812	23.655	-6.022
263	TYR C	8.626	23.680	-6.956	263	TYR O	5.781	24.117	-8.111
263	TYR CB	7.928	21.768	-6.681	263	TYR CG	9.278	23.031	-6.048
263	TYR CD1	10.064	24.846	-6.617	263	TYR CD2	9.600	22.342	-4.998
263	TYR CE1	11.335	24.318	-6.168	263	TYR CE2	11.062	22.640	-4.691
263	TYR CZ	11.838	23.618	-5.186	263	TYR OH	13.083	23.949	-4.897
264	GLY N	4.671	23.161	-6.818	264	GLY CA	3.301	23.904	-7.612
264	GLY C	3.047	22.196	-6.856	264	GLY O	4.047	21.274	-8.361
265	LYS N	3.436	22.477	-8.734	265	LYS CA	3.836	21.798	-10.971
265	LYS C	5.188	22.232	-11.464	265	LYS O	5.084	21.862	-12.386
265	LYS CB	3.783	22.571	-12.944	265	LYS CG	1.600	21.063	-11.308
265	LYS CD	0.716	20.548	-12.879	265	LYS CE	-0.692	20.496	-13.391
265	LYS NZ	-3.678	23.757	-12.489	266	GLY N	5.787	23.226	-10.817
266	GLY CA	7.120	23.633	-11.323	266	GLY C	7.155	25.057	-11.818
266	GLY C	6.177	25.703	-11.648	267	LEU N	8.262	25.336	-12.600
267	LEU CA	8.492	26.642	-13.097	267	LEU C	7.804	26.771	-14.677
267	LEU C	7.913	25.909	-15.298	267	LEU CB	10.010	26.835	-13.216
267	LEU CG	10.432	26.660	-14.858	267	LEU CD1	10.896	29.331	-13.290
267	LEU CD2	11.924	27.923	-14.327	268	ILE N	7.066	27.063	-14.632
268	ILE CA	6.606	28.035	-13.946	268	ILE C	7.426	28.346	-17.065
268	ILE O	8.379	28.793	-16.912	268	ILE CB	8.369	29.210	-15.899
268	ILE CG1	6.099	30.541	-15.962	268	ILE CG2	6.343	26.925	-16.067
268	ILE CD1	5.399	31.765	-16.262	269	ASN N	7.887	27.843	-18.237

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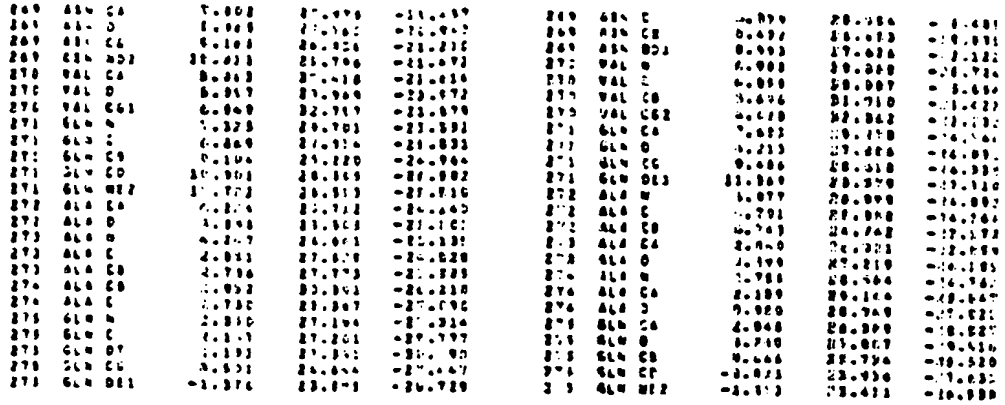

The above structural studies together with the kinetic data presented herein and elsewhere (Philipp, M., et al. (1983) Mol. Cell. Biochem. 51, 5-32; Svendsen, I.B. (1976) Carlsberg Res. Comm. 41, 237-291; Markland, S.F. Id; Stauffe, D.C., et al. (1965) J. Biol. Chem. 244, 5333-5338) indicate that the subsites in the binding cleft of subtilisin are capable of interacting with substrate amino acid residues from P-4 to P-2'.

The most extensively studied of the above residues are Gly166, Gly169 and Ala152. These amino acids were identified as residues within the S-1 subsite. As seen in Fig. 3, which is a stereoview of the S-1 subsite, Gly166 and Gly169 occupy positions at the bottom of the S-1 subsite, whereas Ala152 occupies a position near the top of S-1, close to the catalytic Ser221.

All 19 amino acid substitutions of Gly166 and Gly169 have been made. As will be indicated in the examples which follow, the preferred replacement amino acids for Gly166 and/or Gly169 will depend on the specific amino acid occupying the P-1 position of a given substrate.

The only substitutions of Ala152 presently made and analyzed comprise the replacement of Ala152 with Gly and Ser. The results of these substitutions on P-1 specificity will be presented in the examples.

In addition to those residues specifically associated with specificity for the P-1 substrate amino acid, Tyr104 has been identified as being involved with P-4 specificity. Substitutions at Phe189 and Tyr217, however, are expected to respectively effect P-2' and P-1' specificity.

The catalytic activity of subtilisin has also been modified by single amino acid substitutions at Asn155. The catalytic triad of subtilisin is shown in Fig. 4. As can be seen, Ser221, His64 and Asp32 arc positioned to facilitate nucleophilic attach by the serine hydoxylate on the carbonyl of the scissile peptide bond. Crystallographic studies of subtilisin (Robertus, et al. (1972) Biochem. 11, 4293-4303; Matthews, et al. (1975) J. Biol. Chem. 250, 7120-7126; Poulos, et al. (1976) J. Biol. Chem. 250, 1097-1103) show that two hydrogen bonds are formed with the oxyanion of the substrate transition state. One hydrogen bond donor is from the catalytic serine-221 main-chain amide while the other is from one of the NE2 protons of the asparagine-155 side chain. See Fig. 4.

Asn155 was substituted with Ala, Asp, His, Glu and Thr. These substitutions were made to investigate the the stabilization of the charged tetrahedral intermediate of the transition state complex by the potential hydrogen bond between the side chain of Asn155 and the oxyanion of the intermediate. These particular substitutions caused large decreases in substrate turnover, kcat (200 to 4,000 fold), marginal decreases in substrate binding Km (up to 7 fold), and a loss in transition state stabilization energy of 2.2 to 4.7 kcal/mol. The retention of Km and the drop in kcat will make these mutant enzymes useful as binding proteins for specific; peptide sequences, the nature of which will be determined by the specificity of the precursor protease.

Various other amino acid residues have been identified which affect alkaline stability. In some cases, mutants having altered alkaline stability also have altered thermal stability.

In B amyloliquefaciens subtilisin residues Asp36, Ile107, Lys170, Ser204 and Lys213 have been identified as residues which upon substitution with a different amino acid alter the alkaline stability of the mutated enzyme as compared to the precursor enzyme. The substitution of Asp36 with Ala and the substitution of Lys170 with Glu each resulted in a mutant enzyme having a lower alkaline stability as compared to the wild type subtilisin. When Ile107 was substituted with Val, Ser204 substituted with Cys, Arg or Leu or Lys213 substituted with Arg, the mutant subtilisin had a greater alkaline stability as compared

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to the wild type subtilisin. However, the mutant Ser204P demonstrated a decrease in alkaline stability.

In addition, other residues, identified as being associated with the modification of other properties of subtilisin, also affect alkaline stability. These residues include Ser24, Met50, Glu156, Gly166, Gly169 and Tyr217. Specifically the following particular substitutions result in an increased alkaline stability: Ser24C, Met50F, Gly156Q or S, Gly166A, H, K, N or Q, Gly169S or A, and Tyr217F, K, R or L. The mutant Met50V, on the other hand, results in a decrease in the alkaline stability of the mutant subtilisin as compared to wild type subtilisin.

Other residues involved in alkaline stability based on the alkaline stability screen include Asp197 and Met222. Particular mutants include Asp197(R or A) and Met 222 (all other amino acids).

Various other residues have been identified as being involved in thermal stability as determined by the thermal stability screen herein. These residues include the above identified residues which effect alkaline stability and Met199 and Tyr21. These latter two residues are also believed to be important for alkaline stability. Mutants at these residues include I199 and F21.

The amino acid sequence of B. amyloliquefaciens substilisin has also been modified by substituting two or more amino acids of the wild-type sequence. Six categories of multiply substituted mutant subtilisin have been identified. The first two categories comprise thermally and oxidatively stable mutants. The next three other categories comprise mutants which combine the useful properties of any of several single mutations of B. amyloliquefaciens subtilisin. The last category comprises mutants which have modified alkaline and/or thermal stability.

The first category comprises double mutants in which two cysteine residues have been substituted at various amino acid residue positions within the subtilisin molecule. Formation of disulfide bridges between the two substituted cysteine residues results in mutant subtilisins with altered thermal stability and catalytic activity. These mutants include A21/C22/C87 and C24/C87 which will be described in more detail in Example 11.

The second category of multiple subtilisin mutants comprises mutants which are stable in the presence of various oxidizing agents such as hydrogen peroxide or peracids. Examples 1 and 2 describe these mutants which include F50/I124/Q222, F50/I124, F50/Q222, F50/L124/Q222, I124/Q222 and L124/Q222.

The third category of multiple subtilisin mutants comprises mutants with substitutions at position 222 combined with various substitutions at positions 166 or 169. These mutants, for example, combine the property of oxidative stability of the A222 mutation with the altered substrate specificity of the various 166 or 169 substitutions. Such multiple mutants include A166/A222, A166/C222, F166/C222, K166/A222, K166/C222, V166/A222 and V166/C222. The K166/A222 mutant subtilisin, for example, has a kcat/Km ratio which is approximately two times greater than that of the single A222 mutant subtilisin when compared using a substrate with phenylalanine as the P-1 amino acid. This category of multiple mutant is described in more detail in Example 12.

The fourth category of multiple mutants combines substitutions at position 156 (Glu to Q or S) with the substitution of Lys at position 166. Either of these single mutations improve enzyme performance upon substrates with glutamate as the P-1 amino acid. When these single mutations are combined, the resulting multiple enzyme mutants perform better than either precursor. See Example 9.

The fifth category of multiple mutants contain the substitution of up to four amino acids of the B. amyloliquefaciens subtilisin sequence. These mutants have specific properties which are virtually identicle to the properties of the subtilisin from B. licheniformis. The subtilisin from B. licheniformis differs from B. amyloliquefaciens subtilisin at 87 out of 275 amino acids. The multiple mutant F50/S156/A169/L217 was found to have similar substrate specificity and kinetics to the licheniformis enzyme. (See Example 13.) However, this is probably due to only three of the mutations (S156, A169 and L217) which are present in the substrate binding region of the enzyme. It is quite surprising that, by making only three changes out of the 87 different amino acids between the sequence of the two enzymes, the B. amyloliquifaciens enzyme was converted into an enzyme with properties similar to B. licheniformis enzyme. Other enzymes in this series include F50/Q156/N166/L217 and F50/S156/L217.

The sixth category of multiple mutants includes the combination of substitutions at position 107 (Ile to V) with the substitution of Lys at position 213 with Arg, and the combination of substitutions of position 204 (preferably Ser to C or L but also to all other amino acids) with the substituion of Lys at position 213 with R. Other multiple mutants which have altered alkaline stability include Q156/K166, Q156/N166, S156/K166, S156/N166 (previously identified as having altered substrate specificity), and F50/S156/A169/L217 (previously identified as a mutant of B. amyloliquifaciens subtilisin having properties similar to subtilisin from B. licheniformis). The mutant F50/V107/R213 was constructed based on the observed increase in alkaline stability for the single mutants F50, V107 and R213. It was determined that the V107/R213 mutant had an increased alkaline stability as compared to the wild type subtilisin. In this particular mutant, the increased

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alkaline stability was the result of the cumulative stability of each of the individual mutations. Similarly, the mutant F50/V107/R213 had an even greater alkaline stability as compared to the V107/R213 mutant indicating that the increase in the alkaline stability due to the F50 mutation was also cumulative.

Table IV summarizes the multiple mutants which have been made including those not mentioned above. In addition, based in part on the above results, substitution at the following residues in subtilisin is expected to produce a multiple mutant having increased thermal and alkaline stability: Ser24, Met50, Ile107, Glu156, Gly166, Gly169, Ser204, Lys213, Gly215, and Tyr217.

TABLE IV

Double Mutants	Triple, Quadruple or Other Multiple
C22/C87	F50/I124/Q222
C24/C87	F50/L124/Q222
V45/V48	F50/L124/A222
C49/C94	A21/C22/C87
C49/C95	F50/S156/N166/L217
C50/C95	F50/Q156/N166/L217
C50/C110	F50/S156/A169/L217
F50/I124	F50/S156/L217
F50/Q222	F50/Q156/K166/L217
I124/Q222	F50/S156/K166/L217
Q156/D166	F50/Q156/K166/K217
Q156/K166	F50/S156/K166/K217
Q156/N166	F50/V107/R213
S156/D166 S156/K166	[S153/S156/A158/G159/S160/Δ161-164/I165/S166/A169/R170]
S156/N166	L204/R213
S156/A169 A166/A222 A166/C222	R213/204A, E, Q, D, N, G, K, V, R, T, P, I, M, F, Y, W or H
F166/A222 F166/C222 K166/A222 K166/C222 V166/A222 V166/C222 A169/A222 A169/A222 A169/C222 A21/C22	V107/R213

In addition to the above identified amino acid residues, other amino acid residues of subtilisin are also considered to be important with regard to substrate specificity. Mutation of each of these residues is expected to produce changes in the substrate specificity of subtilisin. Moreover, multiple mutations among these residues and among the previously identified residues are also expected to produce subtilisin mutants having novel substrate specificity.

Particularly important residues are His67, Ile107, Leu126 and Leu135. Mutation of His67 should alter the S-1' subsite, thereby altering the specificity of the mutant for the P-1' substrate residue. Changes at this position could also affect the pH activity profile of the mutant. This residue was identified based on the inventor's substrate modeling from product inhibitor complexes.

Ile107 is involved in P-4 binding. Mutation at this position thus should alter specificity for the P-4 substrate residue in addition to the observed effect on alkaline stability. Ile107 was also identified by molecular modeling from product inhibitor complexes.

The S-2 binding site includes the Leu126 residue. Modification at this position should therefore affect P-2 specificity. Moreover, this residue is believed to be important to convert subtilisin to an amino peptidase.

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The pH activity profile should also be modified by appropriate substitution. These residues were identified from inspection of the refined model, the three dimensional structure from modeling studies. A longer side chain is expected to preclude binding of any side chain at the S-2 subsite. Therefore, binding would be restricted to subsites S-1, S-1', S-2', S-3' and cleavage would be forced to occur after the amino terminal peptide.

Leu135 is in the S-4 subsite and if mutated should alter substrate specificity for P-4 if mutated. This residue was identified by inspection of the three-dimensional structure and modeling based on the product inhibitor complex of F222.

In addition to theses sites, specific amino acid residues within the segments 97-103, 126-129 and 213-215 are also believed to be important to substrate binding.

Segments 97-103 and 126-129 form an antiparallel beta sheet with the main chain of substrate residues P-4 through P-2. Mutating residues in those regions should affect the substrate orientation through main chain (enzyme) - main chain (substrate) interactions, since the main chain of these substrate residues do not interact with these particular residues within the S-4 through S-2 subsites.

Within the segment 97-103, Gly97 and Asp99 may be mutated to alter the position of residues 101-103 within the segment. Changes at these sites must be compatible, however. In B. amyloliquifaciens subtilisin Asp99 stabilizes a turn in the main chain tertiary folding that affects the direction of residues 101-103. B. licheniformis subtilisin Asp97, functions in an analogous manner.

In addition to Gly97 and Asp99, Ser101 interacts with Asp99 in B. amyliquefaciens subtilisin to stabilize the same main chain turn. Alterations at this residue should alter the 101-103 main chain direction. Mutations at Glu103 are also expected to affect the 101-103 main chain direction.

The side chain of Gly102 interacts with the substrate P-3 amino acid. Side chains of substituted amino acids thus are expected to significantly affect specificity for the P-3 substrate amino acids.

All the amino acids within the 127-129 segment are considered important to substrate specificity. Gly127 is positioned such that its side chain interacts with the S-1 and S-3 subsites. Altering this residue thus should alter the specificity for P-1 and P-3 residues of the substrate.

The side chain of Gly128 comprises a part of both the S-2 and S-4 subsites. Altered specificity for P-2 and P-4 therefore would be expected upon mutation. Moreover, such mutation may convert subtilisin into an amino peptidase for the same reasons substitutions of Leu126 would be expected to produce that result.

The Pro129 residue is likely to restrict the conformational freedom of the sequence 126-133, residues which may play a major role in determining P-1 specificity. Replacing Pro may introduce more flexibility thereby broadening the range of binding capabilities of such mutants.

The side chain of Lys213 is located within the S-3 subsite. All of the amino acids within the 213-215 segment are also considered to be important to substrate specificity. Accordingly, altered P-3 substrate specificity is expected upon mutation of this residue.

The Tyr214 residue does not interact with substrate but is positioned such that it could affect the conformation of the hair pin loop 204-217.

Finally, mutation of the Gly215 residue should affect the S-3' subsite, and thereby alter P-3' specificity.

In addition to the above substitutions of amino acids, the insertion or deletion of one or more amino acids within the external loop comprising residues 152-172 may also affect specificity. This is because these residues may play a role in the "secondary contact region" described in the model of streptomyces subtilisin inhibitor complexed with subtilisin. Hirono, et al. (1984) J. Mol. Biol. 178, 389-413. Thermitase K has a deletion in this region, which eliminates several of these "secondary contact" residues. In particular, deletion of residues 161 through 164 is expected to produce a mutant subtilisin having modified substrate specificity. In addition, a rearrangement in this area induced by the deletion should alter the position of many residues involved in substrate binding, predominantly at P-1. This, in turn, should affect overall activity against proteinaceous substrates

The effect of deletion of residues 161 through 164 has been shown by comparing the activity of the wild type (WT) enzyme with a mutant enzyme containing this deletion as well as multiple substitutions (i.e., S153/S156/A158/G159/S160/Δ161-164/I165/S166/A169/R170). This produced the following results:

TABLE V

	kcat	Km	kcat/Km
WT	50	1.4×10^{-4}	3.6×10^{5}
Deletion mutant	8	5.0×10^{-6}	1.6×10^{6}

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The WT has a kcat 6 times greater than the deletion mutant but substrate binding is 28 fold tighter by the deletion mutant. The overall efficiency of the deletion mutant is thus 4.4 times higher than the WT enzyme.

All of these above identified residues which have yet to be substituted, deleted or inserted into are presented in Table VI.

TABLE VI

Substitution/Insertion/Deletion	
Residues	
His67	Ala152
Leu126	Ala153
Leu135	Gly154
Gly97	Asn155
Asp99	Gly156
Ser101	Gly157
Gly102	Gly160
Glu103	Thr158
Leu126	Ser159
Gly127	Ser161
Gly128	Ser162
Pro129	Ser163
Tyr214	Thr164
Gly215	Val165
Gly166	Gly169
Tyr167	Lys170
Pro168	Tyr171
	Pro172

The following disclosure is intended to serve as a representation of embodiments herein, and should not be construed as limiting the scope of this application. These specific examples disclose the construction of certain of the above identified mutants. The construction of the other mutants, however, is apparent from the disclosure herein and that presented in EPO Publication No. 0130756.

All literature citations are expressly incorporated by reference.

EXAMPLE 1

Identification of Peracid Oxidizable Residues of Subtilisin Q222 and L222

As shown in Figures 6A and 6B, organic peracid oxidants inactivate the mutant subtilisins Met222L and Met222Q (L222 and Q222). This example describes the identification of peracid oxidizable sites in these mutant subtilisins.

First, the type of amino acid involved in peracid oxidation was determined. Except under drastic conditions (Means, G.E., et al. (1971) Chemical Modifications of Proteins, Holden-Day, S.F., CA, pp. 160-162), organic peracids modify only methionine and tryptophan in subtilisin. Difference spectra of the enzyme over the 250nm to 350nm range were determined during an inactivation titration employing the reagent, diperdodecanoic acid (DPDA) as oxidant. Despite quantitative inactivation of the enzyme, no change in absorbance over this wavelength range was noted as shown in Figures 7A and 7B indicating that tryptophan was not oxidized. Fontana, A., et al. (1980) Methods in Peptide and Protein Sequence Analysis - (C. Birr ed.) Elsevier, New York, p. 309. The absence of tryptophan modification implied oxidation of one or more of the remaining methionines of B. amyloliquefaciens subtilisin. See Figure 1.

To confirm this result the recombinant subtilisin Met222F was cleaved with cyanogen bromide (CNBr) both before and after oxidation by DPDA. The peptides produced by CNBr cleavage were analyzed on high resolution SDS-pyridine peptide gels (SPG).

Subtilisin Met222F (F222) was oxidized in the following manner. Purified F222 was resuspended in 0.1 M sodium borate pH 9.5 at 10 mg/ml and was added to a final concentration of 26 diperdodecanoic acid

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(DPDA) at 26 mg/ml was added to produce an effective active oxygen concentration of 30 ppm. The sample was incubated for at least 30 minutes at room temperature and then quenched with 0.1 volume of 1 M Tris pH 8.6 buffer to produce a final concentration of 0.1 M Tris pH 8.6). 3mM phenylmethylsulfonyl fluoride (PMSF) was added and 2.5 ml of the sample was applied to a Pharmacia PD10 column equilibrated in 10 mM sodium phosphate pH 6.2, 1 mM PMSF. 3.5 ml of 10 mM sodium phosphate pH6.2, 1mM PMSF was applied and the eluant collected.

F222 and DPDA oxidized F222 were precipitated with 9 volumes of acetone at -20 • C. The samples were resuspended at 10 mg/ml in 8M urea in 88% formic acid and allowed to sit for 5 minutes. An equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein) and the samples incubated for 2 hours at room temperature in the dark. Prior to gel electrophoresis, the samples were lyophilized and resuspended at 2-5 mg/ml in sample buffer (1% pyridine, 5% $NaDodSO_4$, 5% glycerol and bromophenol blue) and disassociated at 95 • C for 3 minutes.

The samples were electrophoresed on discontinuous polyacrylamide gels (Kyte, J., et al. (1953) Anal. Bioch. 133, 515-522). The gels were stained using the Pharmacia silver staining technique (Sammons, D.W., et al. (1981) Electrophoresis 2 135-141).

The results of this experiment are shown in Figure 8. As can be seen, F222 treated with CNBr only gives nine resolved bands on SPG. However, when F222 is also treated with DPDA prior to cleavage, bands X, 7 and 9 disappear whereas bands 5 and 6 are greatly increased in intensity.

In order to determine which of the methionines were effected, each of the CNBr peptides was isolated by reversed phase HPLC and further characterized. The buffer system in both Solvent A (aqueous) and Solvent B (organic) for all HPLC separations was 0.05% triethylamime/trifloroacetic acid (TEA-TFA). In all cases unless noted, solvent A consisted of 0.05% TEA-TFA in H_2O, solvent B was 0.05% TEA-TFA in 1-propanol, and the flow rate was 0.5 ml/minute.

For HPLC analysis, two injections of 1 mg enzyme digest were used. Three samples were acetone precipitated, washed and dried. The dried 1 mg samples were resuspended at 10 mg/ml in 8M urea, 88% formic acid; an equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein). After incubation for 2 hours in the dark at room temperature, the samples were desalted on a 0.8 cm X 7 cm column of Tris Acryl GF05 coarse resin (IBF, Paris, France) equilibrated with 40% solvent B, 60% solvent A. 200 ul samples were applied at a flow rate of 1 ml a minute and 1.0-1.2 ml collected by monitoring the absorbance at 280nm. Prior to injection on the HPLC, each desalted sample was diluted with 3 volumes of solvent A. The samples were injected at 1.0 ml/min (2 minutes) and the flow then adjusted to 0.5 ml/min (100% A). After 2 minutes, a linear gradient to 60% B at 1.0% B/min was initiated. From each 1 mg run, the pooled peaks were sampled (50ul) and analyzed by gel electrophoresis as described above.

Each polypeptide isolated by reversed phase HPLC was further analyzed for homogeneity by SPG. The position of each peptide on the known gene sequence (Wells, J.A., et al. (1983) Nucleic Acids Res. 11 7911-7924) was obtained through a combination of amino acid compositional analysis and, where needed, amino terminal sequencing.

Prior to such analysis the following peptides were to rechromatographed.

1. CNBr peptides from F222 not treated with DPDA:

Peptide 5 was subjected to two additional reversed phase separations. The 10 cm C4 column was equilibrated to 80%A/ 20%B and the pooled sample applied and washed for 2 minutes. Next an 0.5% ml B/min gradient was initiated. Fractions from this separation were again rerun, this time on the 25 cm C4 column, and employing 0.05% TEA-TFA in acetonitrile/1-propanol (1:1) for solvent B. The gradient was identical to the one just described.

Peptide "X" was subjected to one additional separation after the initial chromatography. The sample was applied and washed for 2 minutes at 0.5ml/min (100%A), and a 0.5% ml B/min gradient was initiated.

Peptides 7 and 9 were rechromatographed in a similar manner to the first rerun of peptide 5.

Peptide 8 was purified to homogeneity after the initial separation.

2. CNBr Peptides from DPDA Oxidized F222:

Peptides 5 and 6 from a CNBr digest of the oxidized F222 were purified in the same manner as peptide 5 from the untreated enzyme.

Amino acid compositional analysis was obtained as follows. Samples (-1nM each amino acid) were dried, hydrolyzed in vacuo with 100 ul 6N HCl at 106 • C for 24 hours and then dried in a Speed Vac. The samples were analyzed on a Beckmann 6300 AA analyzer employing ninhydrin detection.

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Amino terminal sequence data was obtained as previously described (Rodriguez, H., et al. (1984) Anal. Biochem. 134, 538-547).

The results are shown in Table VII and Figure 9.

TABLE VII

Amino and COOH terminii of CNBr fragments Terminus and Method		
Fragment	amino, method	COOH, method
X	1, sequence	50, composition
9	51, sequence	119, composition
7	125, sequence	199, composition
8	200, sequence	275, composition
5ox	1, sequence	119, composition
6ox	120, composition	199, composition

Peptides 5ox and 6ox refer to peptides 5 and 6 isolated from CNBr digests of the oxidized protein where their respective levels are enhanced.

From the data in Table VII and the comparison of SPG tracks for the oxidized and native protein digests in Figure 8, it is apparent that (1) Met50 is oxidized leading to the loss of peptides X and 9 and the appearance of 5; and (2) Met124 is also oxidized leading to the loss of peptide 7 and the accumulation of peptide 6. Thus oxidation of B. amyloliquifaciens subtilisin with the peracid, diperdocecanoic acid leads to the specific oxidation of methionine at residues 50 and 124.

EXAMPLE 2

Substitution at Met50 and Met124 in Subtilisin Met222Q

The choice of amino acid for substitution at Met50 was based on the available sequence data for subtilisins from B. licheniformis (Smith, E.C., et al. (1968) J. Biol. Chem. 243, 2184-2191), B.DY (Nedkov, P., et al. (1983) Hoppe Sayler's Z. Physiol. Chem. 364 1537-1540), B. amylosacchariticus (Markland, F.S., et al. (1967) J. Biol. Chem. 242 5198-5211) and B. subtilis (Stahl, M.L., et al. (1984) J. Bacteriol. 158, 411-418). In all cases, position 50 is a phenylalanine. See Figure 5. Therefore, Phe50 was chosen for construction.

At position 124, all known subtilisins possess a methionine. See Figure 5. Molecular modelling of the x-ray derived protein structure was therefore rehired to determine the most probable candidates for substitution. From all 19 candidates, isoleucine and leucine were chosen as the best residues to employ. In order to test whether or not modification at one site but not both was sufficient to increase oxidative stability, all possible combinations were built on the Q222 backbone (F50/Q222, I124/Q222, F50/I124/Q222).

A. Construction of Mutations Between Codons 45 and 50

All manipulations for cassette mutagenesis were carried out on pS4.5 using methods disclosed in EPO Publication No. 0130756 and Wells, J.A., et al, (1985) Gene 34, 315-323. The pΔ50 in Fig. 10, line 4, mutations was produced using the mutagenesis primer shown in Fig. 10, line 6, and employed an approach designated as restriction-purification which is described below. Briefly, a M13 template containing the subtilisin gene, M13mp11-SUBT was used for heteroduplex synthesis (Adelman, et al (1983), DNA 2, 183-193). Following transfection of JM101 (ATCC 33876), the 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was subcloned from M13mp11 SUBT rf into a recipient vector fragment of pBS42 the construction of which is described in EPO Publication No. 0130756. To enrich for the mutant sequence (pΔ50, line 4), the resulting plasmid pool was digested with KpnI, and linear molecules were purified by polyacrylamide gel electrophoresis. Linear molecules were ligated back to a circular form, and transformed into E. coli MM294 cells (ATCC 31446). Isolated plasmids were screened by restriction analysis for the KpnI, site. KpnI$^+$ plasmids were sequenced and confirmed the pΔ50 sequence. Asterisks in Figure 11 indicate the bases that are mutated from the wid type sequence (line 4). pΔ50 (line 4) was cut with StuI and EcoRI and the 0.5 Kb fragment containing the 5' half of the subtilisin gene was purified (fragment 1). pΔ50 (line 4) was digested with KpnI and EcoRI and the 4.0 Kb fragment containing the 3' half of the subtilisin gene and vector sequences was purified (fragment 2). Fragments 1 and 2 (line 5), and duplex DNA

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cassettes coding for mutations desired (shaded sequence, line 6) were mixed in a molar ratio of 1:1:10, respectively. For the particular construction of this example the DNA cassette contained the triplet TTT for codon 50 which encodes Phe. This plasmid was designated pF50. The mutant subtilisin was designated F50.

B. Construction of Mutation Between Codons 122 and 127

The procedure of Example 2A was followed in substantial detail except that the mutagenesis primer of Figure 11, line 7 was used and restriction-purification for the EcoRV site in pΔ124 was used. In addition, the DNA cassette (shaded sequence, Figure 11, line 6) contained the triplet ATT for codon 124 which encodes Ile and CTT for Leu. Those plasmids which contained the substitution of Ile for Met124were designeated pI124. The mutant subtilisin was designated I124.

C. Construction of Various F50/I124/Q222 Multiple Mutants

The triple mutant, F50/I124/Q222, was constructed from a three-way ligation in which each fragment contained one of the three mutations. The single mutant Q222 (pQ222) was prepared by cassette mutagenesis as described in EPO Publication No. 0130756. The F50 mutation was contained on a 2.2kb AvaII to PvuII fragment from pF50; the I124 mutation was contained on a 260 bp PvuII to AvaII fragment from pI124; and the Q222 mutation was contained on 2.7 kb AvaII to AvaII fragment from pQ222. The three fragments were ligated together and transformed into E. coli MM294 cells. Restriction analysis of plasmids from isolated transformants confirmed the construction. To analyze the final construction it was convenient that the AvaII site at position 798 in the wild-type subtilisin gene was eliminated by the I124 construction.

The F50/Q222 and I124/Q222 mutants were constructed in a similar manner except that the appropriate fragment from pS4.5 was used for the final construction.

D. Oxidative Stability of Q222 Mutants

The above mutants were analyzed for stability to peracid oxidation. As shown in Fig. 12, upon incubation with diperdodecanoic acid (protein 2mg/mL, oxidant 75ppm[0]), both the I124/Q222 and the F50/I124/Q222 are completely stable whereas the F50/Q222 and the Q222 are inactivated. This indicates that conversion of Met124 to I124 in subtilisin Q222 is sufficient to confer resistance to organic peracid oxidants.

EXAMPLE 3

Subtilisin Mutants Having Altered Substrate Specificity-Hydrophobic Substitutions at Residues 166

Subtilisin contains an extended binding cleft which is hydrophobic in character. A conserved glycine at residue 166 was replaced with twelve non-ionic amino acids which can project their side-chains into the S-1 subsite. These mutants were constructed to determine the effect of changes in size and hydrophobicity on the binding of various substrates.

A. Kinetics for Hydrolysis of Substrates Having Altered P-1 Amino Acids by Subtilisin from B. Amyloliquefaciens

Wild-type subtilisin was purified from B. subtilis culture supernatants expressing the B. amyloliquefaciens subtilisin gene (Wells, J.A., et al. (1983) Nucleic Acids Res. 11, 7911-7925) as previously described (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Details of the synthesis of tetrapeptide substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide (where X is the P1 amino acid) are described by DelMar, E.G., et al. (1979) Anal. Biochem. 99, 316-320. Kinetic parameters, Km(M) and kcat-(s^{-1}) were measured using a modified progress curve analysis (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Briefly, plots of rate versus product concentration were fit to the differential form of the rate equation using a non-linear regression algorithm. Errors in kcat and Km for all values reported are less than five percent. The various substrates in Table VIII are ranged in order of decreasing hydrophobicity. Nozaki, Y. (1971), J. Biol. Chem. 246, 2211-2217; Tanford C. (1978) Science 200, 1012).

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TABLE VIII

P1 substrate Amino Acid	kcat(S^{-1})	1/Km(M^{-1})	kcat/Km ($s^{-1}M^{-1}$)
Phe	50	7,100	360,000
Tyr	28	40,000	1,100,000
Leu	24	3,100	75,000
Met	13	9,400	120,000
His	7.9	1,600	13,000
Ala	1.9	5,500	11,000
Gly	0.003	8,300	21
Gln	3.2	2,200	7,100
Ser	2.8	1,500	4,200
Glu	0.54	32	16

The ratio of kcat/Km (also referred to as catalytic efficienty) is the apparent second order rate constant for the conversion of free enzyme plus substrate (E + S) to enzyme plus products (E + P) (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287). The log (kcat/Km) is proportional to transition state binding energy, ΔG_T^* . A plot of the log kcat/Km versus the hydrophobicity of the P1 side-chain (Figure 14) shows a strong correlation (r = 0.98), with the exception of the glycine substrate which shows evidence for non-productive binding. These data show that relative differences between transition-state binding energies can be accounted for by differences in P-1 side-chain hydrophobicity. When the transition-state binding energies are calculated for these substrates and plotted versus their respective side-chain hydrophobicities, the line slope is 1.2 (not shown). A slope greater than unity, as is also the case for chymotrypsin (Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Harper, J.W., et al. (1984) Biochemistry, 23, 2995-3002), suggests that the P1 binding cleft is more hydrophobic than ethanol or dioxane solvents that were used to empirically determine the hydrophobicity of amino acids (Nozaki, Y., et al. J. Biol. Chem. (1971) 246, 2211-2217; Tanford, C. (1978) Science 200, 1012).

For amide hydrolysis by subtilisin, kcat can be interpreted as the acylation rate constant and Km as the dissociation constant, for the Michaelis complex (E•S), Ks. Gutfreund, H., et al (1956) Biochem. J. 63, 656. The fact that the log kcat, as well as log 1/Km, correlates with substrate hydrophobicity is consistent with proposals (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303) that during the acylation step the P-1 side-chain moves deeper into the hydrophobic cleft as the substrate advances from the Michaelis complex (E•S) to the tetrahedral transition-state complex (E•S*). However, these data can also be interpreted as the hydrophobicity of the P1 side-chain effecting the orientation, and thus the susceptibility of the scissile peptide bond to nucleophilic attack by the hydroxyl group of the catalytic Ser221.

The dependence of kcat/Km on P-1 side chain hydrophobicity suggested that the kcat/Km for hydrophobic substrates may be increased by increasing the hydrophobicity of the S-1 binding subsite. To test this hypothesis, hydrophobic amino acid substitutions of Gly166 were produced.

Since hydrophobicity of aliphatic side-chains is directly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927), increasing the hydrophobicity in the S-1 subsite may also sterically hinder binding of larger substrates. Because of difficulties in predicting the relative importance of these two opposing effects, we elected to generate twelve non-charged mutations at position 166 to determine the resulting specificities against non-charged substrates of varied size and hydrophobicity.

B. Cassette Mutagenesis of the P1 Binding Cleft

The preparation of mutant subtilisims containing the substitution of the hydrophobic amino acids Ala, Val and Phe into residue 166 has been described in EPO Publication No. 0130756. The same method was used to produce the remaining hydrophobic mutants at residue 166. In applying this method, two unique and silent restriction sites were introduced in the subtilisin genes to closely flank the target codon 166. As can be seen in Figure 13, the wild type sequence (line 1) was altered by site-directed mutagenesis in M13 using the indicated 37mer mutagenesis primer, to introduce a 13 bp delection (dashedline) and unique SacI and XmaI sites (underlined sequences) that closely flank codon 166. The subtilisin gene fragment was subcloned back into the E. coli - B. subtilis shuttle plasmid, pBS42, giving the plasmid pΔ166 (Figure 13,

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line 2). pΔ166 was cut open with SacI and XmaI, and gapped linear molecules were purified (Figure 13, line 3). Pools of synthetic oligonucleotides containing the mutation of interest were annealed to give duplex DNA cassettes that were ligated into gapped pΔ166 (underlined and overlined sequences in Figure 13, line 4). This construction restored the coding sequence except over position 166(NNN; line 4). Mutant sequences were confirmed by dideoxy sequencing. Asterisks denote sequence changes from the wild type sequence. Plasmids containing each mutant B. amyloliquefaciens subtilisin gene were expressed at roughly equivalent levels in a protease deficient strain of B. subtilis, BG2036 as previously described. EPO Publication No. 0130756; Yang, M., et al. (1984) J. Bacteriol. 160, 15-21; Estell, D.A., et al (1985) J. Biol. Chem. 260, 6518-6521.

C. Narrowing Substrate Specificity by Steric Hindrance

To probe the change in substrate specificity caused by steric alterations in the S-1 subsite, position 166 mutants were kinetically analyzed versus P1 substrates of increasing size (i.e., Ala, Met, Phe and Tyr). Ratios of kcat/Km are presented in log form in Figure 15 to allow direct comparisons of transition-state binding energies between various enzyme-substrate pairs.

According to transition state theory, the free enery difference between the free enzyme plus substrate (E + S) and the transition state complex (E•S*) can be calculated from equation (1),

$$(1) \quad {}^{\Delta}G_T^{\neq} = -RT \ln kcat/Km + RT \ln kT/h$$

in which kcat is the turnover number, Km is the Michaelis constant, R is the gas constant, T is the temperature, k is Boltzmann's constant, and h is Planck's constant. Specificity differences are ezpressed quantitatively as differences between transition state binding energies (i.e., $\Delta\Delta G_t^{*}$), and can be calculated from equation (2).

$$(2) \quad {}^{\Delta\Delta}G_T^{\neq} = -RT \ln (kcat/Km)_A/(kcat/Km)_B$$

A and B represent either two different substrates assayed againt the same enzyme, or two mutant enzymes assayed against the same substrate.

As can be seen from Figure 15A, as the size of the side-chain at position 166 increases the substrate preference shifts from large to small P-1 side-chains. Enlarging the side-chain at position 166 causes kcat/Km to decrease in proportion to the size of the P-1 substrate side-chain (e.g., from Gly166 (wild-type) through W166, the kcat/Km for the Tyr substrate is decreased most followed in order by the Phe, Met and Ala P-1 substrates).

Specific steric changes in the position 166 side-chain, such as he presence of a β-hydroxyl group, β- or γ-aliphatic branching, cause large decreases in kcat/Km for larger P1 substrates. Introducing a β-hydroxyl group in going from A166 (Figure 15A) to S166 (Figure 15B), causes an 8 fold and 4 fold reduction in kcat/Km for Phe and Tyr substrates, respectively, while the values for Ala and Met substrates are unchanged. Producing a β-branched structure, in going from S166 to T166, results in a drop of 14 and 4 fold in kcat/Km for Phe and Tyr, respectively. These differences are slightly magnified for V166 which is slightly larger and isosteric with T166. Enlarging the β-branched substituents from V166 to I166 causes a lowering of kcat/Km between two and six fold toward Met, Phe and Tyr substrates. Inserting a γ-branched structure, by replacing M166 (Figure 15A) with L166 (Figure 15B), produces a 5 fold and 18 fold decrease in kcat/Km for Phe and Tyr substrates, respectively. Aliphatic γ-branched appears to induce less steric hindrance toward the Phe P-1 substrate than β-branching, as evidenced by the 100 fold decrease in kcat/Km for the Phe substrate in going from L166 to I166.

Reductions in kcat/Km resulting from increases in side chain size in the S-1 subsite, or specific structural features such as β- and γ-branching, are quantitatively illustrated in Figure 16. The kcat/Km values for the position 166 mutants determined for the Ala, Met, Phe, and Tyr P-1 substrates (top panel through bottom panel, respectively), are plotted versus the position 166 side-chain volumes (Chothia, C. (1984) Ann. Rev. Biochem. 53, 537-572). Catalytic efficiency for the Ala substrate reaches a maximum for

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I166, and for the Met substrate it reaches a maximum between V166 and L166. The Phe substrate shows a broad kcat/Km peak but is optimal with A166. Here, the β-branched position 166 substitutions form a line that is parallel to, but roughly 50 fold lower in kcat/Km than side-chains of similar size [i.e., C166 versus T166, L166 versus I166). The Tyr substrate is most efficiently utilized by wild type enzyme (Gly166), and there is a steady decrease as one proceeds to large position 166 side-chains. The β-branched and γ-branched substitutions form a parallel line below the other non-charged substitutions of similar molecular volume.

The optimal substitution at position 166 decreases in volume with increasing volume of the P1 substrate [i.e., I166/Ala substrate, L166/Met substrate, A166/Phe substrate, Gly166/Tyr substrate]. The combined volumes for these optimal pairs may approximate the volume for productive binding in the S-1 subsite. For the optimal pairs, Gly166/Tyr substrate, A166/Phe substrate, L166/Met substrate, V166/Met substrate, and I166/Ala substrate, the combined volumes are 266,295,313,339 and 261 A^3, respectively. Subtracting the volume of the peptide backbone from each pair (i.e., two times the volume of glycine), an average side-chain volume of 160±32A^3 for productive binding can be calculated.

The effect of volume, in excess to the productive binding volume, on the drop in transition-state binding energy can be estimated from the Tyr substrate curve (bottom panel, Figure 16), because these data, and modeling studies (Figure 2), suggest that any substitution beyond glycine causes steric repulsion. A best-fit line drawn to all the data (r = 0.87) gives a slope indicating a loss of roughly 3 kcal/mol in transition state binding energy per 100A^3 of excess volume. (100A^3 is approximately the size of a leucyl side-chain.)

D. Enhanced Catalytic Efficiency Correlates with Increasing Hydrophobicity of the Position 166 Substitution

Substantial increases in kcat/Km occur with enlargement of the position 166 side-chain, except for the Tyr P-1 substrate (Figure 16). For example, kcat/Km increases in progressing from Gly166 to I166 for the Ala substrate (net of ten-fold), from Gly166 to L166 for the Met substrate (net of ten-fold) and from Gly166 to A166 for the Phe substrate (net of two-fold). The increases in kcat/Km cannot be entirely explained by the attractive terms in the van der Waals potential energy function because of their strong distance dependence ($1/r^6$) and because of the weak nature of these attractive forces (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Levitt, M. (1976) J. Mol. Biol. 104, 59-107). For example, Levitt (Levitt, M. (1976) J. Mol. Biol. 104, 59-107) has calculated that the van der Waals attraction between two methionyl residues would produce a maximal interaction energy of roughly -0.2 kcal/mol. This energy would translate to only 1.4 fold increase in kcat/Km.

The increases of catalytic efficiency caused by side-chain substitutions at position 166 are better accounted for by increases in the hydrophobicity of the S-1 subsite. The increase kcat/Km observed for the Ala and Met substrates with increasing position 166 side-chain size would be expected, because hydrophobicity is roughly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927).

Another example that can be interpreted as a hydrophobic effect is seen when comparing kcat/Km for isosteric substitutions that differ in hydrophobicity such as S166 and C166 (Figure 16). Cysteine is considerably more hydrophobic than serine (-1.0 versus +0.3 kcal/mol) (Nozaki, Y., et al. (1971) J. Biol. Chem. 246, 2211-2217; Tanford, C. (1978) Science 200, 1012). The difference in hydrophobicity correlates with the observation that C166 becomes more efficient relative to Ser166 as the hydrophobicity of the substrates increases (i.e., Ala < Met < Tye < Phe). Steric hindrance cannot explain these differences because serine is considerably smaller than cysteine (99 versus 118A^3). Paul, I.C., Chemistry of the -SH Group (ed. S. Patai, Wiley Interscience, New York, 1974) pp. 111-149.

E. Production of an Elastase-Like Specificity in Subtilisin

The I166 mutation illustrates particularly well that large changes in specificity can be produced by altering the structure and hydrophobicity of the S-1 subsite by a single mutation (Figure 17). Progressing through the small hydrophobic substrates, a maximal specificity improvement over wild type occurs for the Val substrate (16 fold in kcat/Km). As the substrate side chain size increases, these enhancements shrink to near unity (i.e., Leu and His substrates). The I166 enzyme becomes poorer against larger aromatic substrates of increasing size (e.g., I166 is over 1,000 fold worse against the Tyr substrate than is Gly166). We interpret the increase in catalytic efficiency toward the small hydrophobic substrates for I166 compared to Gly166 to the greater hydrophobicity of isoluecine (i.e., -1.8 kcal/mol versus 0). Nozaki, Y., et al. (1971) J. Biol. Chem. 246, 2211-2217; Tanford, C. (1978) Science 200, 1012. The decrease in catalytic efficiency

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toward the very large substrates for I166 versus Gly166 is attributed to steric repulsion.

The specificity differences between Gly166 and I166 are similar to the specificity differences between chymotrypsin and the evolutionary relative, elastase (Harper, J.W., et al (1984) Biochemistry 23, 2995-3002). In elastase, the bulky amino acids, Thr and Val, block access to the P-1 binding site for large hydrophobic substrates that are preferred by chymotrypsin. In addition, the catalytic efficiencies toward small hydrophobic substrates are greater for elastase than for chymotrypsin as we obeseve for I166 versus Gly166 in subtilisin.

EXAMPLE 4

Substitution of Ionic Amino Acids for Gly166

The construction of subtilisin mutants containing the substitution of the ionic amino acids Asp, Asn, Gln, Lys and Ang are disclosed in EPO Publication No. 0130756. The present example describes the construction of the mutant subtilisin containing Glu at position 166 (E166) and presents substrate specificity data on these mutants. Further data on position 166 and 156 single and double mutants is presented infra.

pΔ166, described in Example 3, was digested with SacI and XmaI. The double strand DNA cassette (underlined and overlined) of line 4 in Figure 13 contained the triplet GAA for the codon 166 to encode the replacement of Glu for Gly166. This mutant plasmid designated pQ168 was propagated in BG2036 as described. This mutant subtilisin, together with the other mutants containing ionic substituent amino acids at residue 166, were isolated as described and further analyzed for variations in substrate specificity.

Each of these mutants was analyzed with the tetrapeptide substrates, succinyl-L-AlaL-AlaProL-X-p-nitroanilide, where X was Phe, Ala and Glu.

The results of this analysis are shown in Table IX.

TABLE IX

Position 166	P-1 Substrate (kcat/Km x 10^{-4})		
	Phe	Ala	Glu
Gly (wild type)	36.0	1.4	0.002
Asp (D)	0.5	0.4	<0.001
Glu (E)	3.5	0.4	<0.001
Asn (N)	18.0	1.2	0.004
Gln (Q)	57.0	2.6	0.002
Lys (K)	52.0	2.8	1.2
Arg (R)	42.0	5.0	0.08

These results indicate that charged amino acid substitutions at Gly166 have improved catalytic efficiencies (kcat/Km) for oppositely charged P-1 substrates (as much as 500 fold) and poorer catalytic efficiency for like charged P-1 substrates.

EXAMPLE 5

Substitution of Glycine at Position 169

The substitution of Gly169 in B. amyloliquefaciens subtilisin with Ala and Ser is described in EPO Publication No. 0130756. The same method was used to make the remaining 17 mutants containing all other substituent amino acids for position 169.

The construction protocol is summarized in Figure 18. The overscored and underscored double stranded DNA cassettes used contained the following triplet encoding the substitution of the indicated amino acid at residue 169.

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GCT	A	ATG	M
TGT	C	AAC	N
GAT	D	CCT	P
GAA	E	CAA	Q
TTC	F	AGA	R
GGC	G	AGC	S
CAC	H	ACA	T
ATC	I	GTT	V
AAA	K	TGG	W
CTT	L	TAC	Y

Each of the plasmids containing a substituted Gly169 was designated pX169, where X represents the substituent amino acid. The mutant subtilisins were simialrly designated.

Two of the above mutant subtilisins, A169 and S169, were analyzed for substrate specificity against synthetic substrates containing Phe, Leu, Ala and Arg in the P-1 position. The following results are shown in Table X.

TABLE X

Effect of Serine and Alanine Mutations at Position 169 on P-1 Substrate Specificity				
Position 169	P-1 Substrate [kcat/Km x 10^{-4})			
	Phe	Leu	Ala	Arg
Gly (wild type)	40	10	1	0.4
A169	120	20	1	0.9
S169	50	10	1	0.6

These results indicate that substitutions of Ala and Ser at Gly169 have remarkably similar catalytic efficiencies against a range of P-1 substrates compared to their position 166 counterparts. This is probably because position 169 is at the bottom of the P-1 specificity subsite.

EXAMPLE 6

Substitution at Position 104

Tyr104 has been substituted with Ala, His, Leu, Met and Ser. The method used was a modification of the site directed mutagenesis method. According to the protocol of Figure 19, a primer (shaded in line 4) introduced a unique HindIII site and a frame shift mutation at codon 104. Restriction-purification for the unique HindIII site facilitated the isolation of the mutant sequence (line 4). Restriction-selection against this HindIII site using pimers in line 5 was used to obtain position 104 mutants.

The following triplets were used in the primers of Figure 19, line 5 for the 104 codon which substituted the following amino acids.

GCT	A	TTC	F
ATG	M	CCT	P
CTT	L	ACA	T
AGC	S	TGG	W
CAC	H	TAC	Y
CAA	Q	GTT	V
GAA	E	AGA	R
GGC	G	AAC	N
ATC	I	GAT	D
AAA	K	TGT	C

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The substrates in Table XI were used to analyze the substrate specificity of these mutants. The results obtained fo H104 subtilisin are shown in Table XI.

TABLE XI

Substrate	kcat		Km		Kcat/Km	
	WT	H104	WT	H104	WT	H104
sAAPFpNA	50.0	22.0	$1.4x10^{-4}$	$7.1x10^{-4}$	$3.6x10^{5}$	$3.1x10^{4}$
sAAPApNA	3.2	2.0	$2.3x10^{-4}$	$1.9x10^{-3}$	$1.4x10^{4}$	$1x10^{3}$
sFAPFpNA	26.0	38.0	$1.8x10^{-4}$	$4.1x10^{-4}$	$1.5x10^{5}$	$9.1x10^{4}$
sFAPApNA	0.32	2.4	$7.3x10^{-5}$	$1.5x10^{-4}$	$4.4x10^{3}$	$1.6x10^{4}$

From these data it is clear that the substitution of His for Tyr at position 104 produces an enzyme which is more efficient (higher kcat/Km) when Phe is at the P-4 substrate position than when Ala is at the P-4 substrate position.

EXAMPLE 7

Substitution of Ala152

Ala152 has been substituted by Gly and Ser to determine the effect of such substitutions on substrate specificity.

The wild type DNA sequence was mutated by the V152/P153 primer (Figure 20, line 4) using the above restriction-purification approach for the new KpnI site. Other mutant primers (shaded sequences Figure 20; S152, line 5 and G152, line 6) mutated the new KpnI site away and such mutants were isolated using the restriction-selection procedure as described above for loss of the KpnI site.

The results of these substitutions for the above synthetic substrates containing the P-1 amino acids Phe, Leu and Ala are shown in Table XII.

TABLE XII

Position 152	P-1 Substrate (kcat/Kmx10^{-4})		
	Phe	Leu	Ala
Gly (G)	0.2	0.4	<0.04
Ala (wild type)	40.0	10.0	1.0
Ser (S)	1.0	0.5	0.2

These results indicate that, in contrast to positions 166 and 169, replacement of Ala152 with Ser or Gly causes a dramatic reduction in catalytic efficiencies across all substrates tested. This suggests Ala152, at the top of the S-1 subsite, may be the optimal amino acid because Ser end Gly ore homologous Ala substitutes.

EXAMPLE 8

Substitution at Position 156

Mutants containing the substitution of Ser and Gln for Glu156 have been constructed according to the overall method depicted in Figure 21. This method was designed to facilitate the construciton of multiple mutants at position 156 and 166 as will be described hereinafter. However, by regenerating the wild type Gly166, single mutations at Glu156 were obtained.

The plasmid pΔ166 is already depicted in line 2 of Figure 13. The synthetic oligonucleotides at the top right of Figure 21 represent the same DNA cassettes depicted in line 4 of Figure 13. The plasmid p166 in Figure 21 thus represents the mutant plasmids of Examples 3 and 4. In this particular example, p166 contains the wild type Gly166.

Construction of position 156 single mutants were prepared by ligation of the three fragments (1-3) indicated at the bottom of Figure 21. Fragment 3, containing the carboxy-terminal portion of the subtilisin gene including the wild type position 166 codon, was isolated as a 610 bp SacI-BamHI fragment. Fragment 1 contained the vector sequences, as well as the amino-terminal sequences of the subtilisin gene through codon 151. To produce fragment 1, a unique KpnI site at codon 152 was introduced into the wild type subtilisin sequence from pS4.5. Site-directed mutagenesis in M13 employed a primer having the sequence 5'-TA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3' to produce the mutation. Enrichment for the mutant sequence was accomplished by restriction with KpnI, purification and self ligation. The mutant sequence containing the KpnI site was confirmed by direct plasmid sequencing to give pV152. pV152 (~1 µg) was digested with KpnI and treated with 2 units of DNA polymerase I large fragment (Klenow fragment from Boeringer-Mannheim) plus 50 µM deoxynucleotide triphosphates at 37°C for 30 min. This created a blunt end that terminated with codon 151. The DNA was extracted with 1:1 volumes phenol and $CHCl_3$ and DNA in the aqueous phase was precipitated by addition of 0.1 volumes 5M ammonium acetate and two volumes ethanol. After centrifugation and washing the DNA pellet with 70% ethanol, the DNA was lyophilized. DNA was digested with BamHI and the 4.6kb piece (fragment 1) was purified by acrylamide gel electrophoresis followed by electroelution. Fragment 2 was a duplex synthetic DNA cassette which when ligated with fragments 1 and 3 properly restored the coding sequence except at codon 156. The top strand was synthesized to contain a glutamine codon, and the complementary bottom strand coded for serine at 156. Ligation of heterophosphorylated cassettes leads to a large and favorable bias for the phosphorylated over the non-phosphorylated oligonucleotide sequence in the final segrated plasmid product. Therefore, to obtain Q156 the top strand was phosphorylated, and annealed to the non-phosphorylated bottom strand prior to ligation. Similarly, to obtain S156 the bottom strand was phosphorylated and annealed to the non-phosphorylated top strand. Mutant sequences were isolated after ligation and transformation, and were confirmed by restriction analysis and DNA sequencing as before. To express variant subtilisins, plasmids were transformed into a subtilisin-neutral protease deletion mutant of B. subtilis, BG2036, as previously described. Cultures were fermented in shake flasks for 24 h at 37°C in LB media containing 12.5 mg/mL chloramphenicol and subtilisin was purified from culture supernatants as described. Purity of subtilisin was greater than 95% as judged by SDS PAGE.

These mutant plasmids designated pS156 and pQ156 and mutant subtilisins designated S156 and Q156 were analyzed with the above synthetic substrates where P-1 comprised the amino acids Glu, Gln, Met and Lys. The results of this analyses are presented in Example 9.

EXAMPLE 9

Multiple Mutants With Altered Substrate Specificity - Substitution at Positions 156 and 166

Single substitutions of position 166 are described in Examples 3 and 4. Example 8 describes single substitutions at position 156 as well as the protocol of Figure 21 whereby various double mutants comprising the substitution of various amino acids at positions 156 and 166 can be made. This example describes the construction and substrate specificity of subtilisin containing substitutions at position 156 and 166 and summarizes some of the data for single and double mutants at positions 156 and 166 with various substrates.

K166 is a common replacement amino acid in the 156/166 mutants described herein. The replacement of Lys for Gly166 was achieved by using the synthetic DNA cassette at the top right of Figure 21 which contained the triplet AAA for NNN. This produced fragment 2 with Lys substituting for Gly166.

The 156 substituents were Gln and Ser. The Gln and Ser substitutions at Gly156 are contained within fragment 3 (bottom right Figure 21).

The multiple mutants were produced by combining fragments 1, 2 and 3 as described in Example 8. The mutants Q156/K166 and S156/K166 were selectively generated by differential phosphorylation as described. Alternatively, the double 156/166 mutants, c.f. Q156/K166 and S156/K166, were prepared by ligation of the 4.6kb SacI-BamHI fragment from the relevant p156 plasmid containing the 0.6kb SacI-BamHI fragment from the relevant p166 plasmid.

These mutants, the single mutant K166, and the S156 and Q156 mutants of Example 8 were analyzed for substitute specificity against synthetic polypeptides containing Phe or Glu as the P-1 substrate residue. The results are presented in Table XIII.

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TABLE XIII

Enzymes Compared[b]	Substrate P-1 Residue	kcat	Km	kcat/Km	kcat/Km (mutant) / kcat/Km(wt)
Glu156/Gly166 (WT)	Phe	50.00	1.4×10^{-4}	3.6×10^{5}	(1)
	Glu	0.54	3.4×10^{-2}	1.6×10^{1}	(1)
K166	Phe	20.00	4.0×10^{-5}	5.2×10^{5}	1.4
	Glu	0.70	5.6×10^{-5}	1.2×10^{4}	750
Q156/K166	Phe	30.00	1.9×10^{-5}	1.6×10^{6}	4.4
	Glu	1.60	3.1×10^{-5}	5.0×10^{4}	3100
S156/K166	Phe	30.00	1.8×10^{-5}	1.6×10^{6}	4.4
	Glu	0.60	3.9×10^{-5}	1.6×10^{4}	1000
S156	Phe	34.00	4.7×10^{-5}	7.3×10^{5}	2.0
	Glu	0.40	1.8×10^{-3}	1.1×10^{2}	6.9
E156	Phe	48.00	4.5×10^{-5}	1.1×10^{6}	3.1
	Glu	0.90	3.3×10^{-3}	2.7×10^{2}	17

As can be seen in Table XIV, either of these single mutations improve enzyme performance upon substrates with glutamate at the P-1 enzyme binding site. When these single mutations were combined, the resulting multiple enzyme mutants are better than either parent. These single or multiple mutations also alter the relative pH activity profiles of the enzymes as shown in Figure 23.

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To isolate the contribution of electrostatics to substrate specificity from other chemical binding forces, these various single and double mutants were analyzed for their ability to bind and cleave synthetic substrates containing Glu, Gln, Met and Lys as the P-1 substrate amino acid. This permitted comparisons between side-chains that were more sterically similar but differed in charge (e.g., Glu versus Gln, Lys versus Met). Similarly, mutant enzymes were assayed against homologous P-1 substrates that were most sterically similar but differed in charge (Table XIV).

TABLE XIV

Kinetics of Position 156/166 Subtilisins Determined for Different P1 Substrates

Enzyme Position (a)		Net Charge (b)	P-1 Substrate log kcat/Km (log 1/Km) (c)			
			Glu	Gln	Met	Lys
156	166					
Glu	Asp	-2	n.d.	3.02 (2.56)	3.93 (2.74)	4.23 (3.00)
Glu	Glu	-2	n.d.	3.06 (2.91)	3.86 (3.28)	4.48 (3.69)
Glu	Asn	-1	1.62 (2.22)	3.85 (3.14)	4.99 (3.85)	4.15 (2.88)
Glu	Gln	-1	1.20 (2.12)	4.36 (3.64)	5.43 (4.36)	4.10 (3.15)
Gln	Asp	-1	1.30 (1.79)	3.40 (3.08)	4.94 (3.87)	4.41 (3.22)
Ser	Asp	-1	1.23 (2.13)	3.41 (3.09)	4.67 (3.68)	4.24 (3.07)
Glu	Met	-1	1.20 (2.30)	3.89 (3.19)	5.64 (4.83)	4.70 (3.89)
Glu	Ala	-1	n.d.	4.34 (3.55)	5.65 (4.46)	4.90 (3.24)
Glu	Gly(wt)	-1	1.20 (1.47)	3.85 (3.35)	5.07 (3.97)	4.60 (3.13)
Gln	Gly	0	2.42 (2.48)	4.53 (3.81)	5.77 (4.61)	3.76 (2.82)
Ser	Gly	0	2.31 (2.73)	4.09 (3.68)	5.61 (4.55)	3.46 (2.74)
Gln	Asn	0	2.04 (2.72)	4.51 (3.76)	5.79 (4.66)	3.75 (2.74)
Ser	Asn	0	1.91 (2.78)	4.57 (3.82)	5.72 (4.64)	3.68 (2.80)
Glu	Arg	0	2.91 (3.30)	4.26 (3.50)	5.32 (4.22)	3.19 (2.80)
Glu	Lys	0	4.09 (4.25)	4.70 (3.88)	6.15 (4.45)	4.23 (2.93)
Gln	Lys	+1	4.70 (4.50)	4.64 (3.68)	5.97 (4.68)	3.23 (2.75)
Ser	Lys	+1	4.21 (4.40)	4.84 (3.94)	6.16 (4.90)	3.73 (2.84)
Maximum difference: log kcat/Km (log 1/Km) (d)			3.5 (3.0)	1.8 (1.4)	2.3 (2.2)	-1.3 (-1.0)

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Footnotes to Table XIV:

(a) B. subtilis, BG 2036, expressing indicated variant subtilisin were fermented and enzymes purified as previously described (Estell, et al. (1985) J. Biol. Chem. 260, 6518-6521). Wild type subtilisin is indicated (wt) containing Glu156 and Gly166.

(b) Net charge in the P-1 binding site is defined as the sum of charges from positions 156 and 166 at pH 8.6.

(c) Values for kcat(s^{-1}) and Km(M) were measured in 0.1M Tris pH 8.6 at 25°C as previously described[3] against P-1 substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide, where X is the indicated P-1 amino acid. Values for log 1/Km are shown inside parentheses. All errors in determination of kcat/Km and 1/Km are below 5%.

(d) Because values for Glu156/Asp166(D166) are too small to determine accurately, the maximum difference taken for GluP-1 substrate is limited to a charge range of +1 to -1 charge change.

n.d. = not determined

The kcat/Km ratios shown are the second order rate constants for the conversion of substrate to product, and represent the catalytic efficiency of the enzyme. These ratios are presented in logarithmic form to scale the data, and because log kcat/Km is proportional to the lowering of transition-state activation energy (ΔG_T). Mutations at position 156 and 166 produce changes in catalytic efficiency toward Glu, Gln, Met and Lys P-1 substrates of 3100, 60, 200 and 20 fold, respectively. Making the P-1 binding-site more positively charged [e.g., compare Gln156/Lys166 (Q156/K166) versus Glu156/Met166 (Glu156/M166)] dramatically increased kcat/Km toward the Glu P-1 substrate (up to 3100 fold), and decreased the catalytic efficiency toward the Lys P-1 substrate (up to 10 fold). In addition, the results show that the catalytic efficiency of wild type enzyme can be greatly improved toward any of the four P-1 substrates by mutagenesis of the P-1 binding site.

The changes in kcat/Km ore caused predominantly by changes in 1/Km. Because 1/Km is approximately equal to 1/Ks, the enzyme-substrate association constant, the mutations primarily cause a change in substrate binding. These mutations produce smaller effects on kcat that run parallel to the effects on 1/Km. The changes in kcat suggest either an alteration in binding in the P-1 binding site in going from the Michaelis-complex E•S) to the transition-state complex (E-S*) as previously proposed (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), or change in the position of the scissile peptide bond over the catalytic serine in the E•S complex.

Changes in substrate preference that arise from changes in the net charge in the P-1 binding site show trends that are best accounted for by electrostatic effects (Figure 28). As the P-1 binding cleft becomes more positively charged, the average catalytic efficiency increases much more for the Glu P-1 substrate than for its neutral and isosteric P-1 homolog, Gln (Figure 28A). Furthermore, at the positive extreme both substrates have nearly identical catalytic efficiencies.

In contrast, as the P-1 site becomes more positively charged the catalytic efficiency toward the Lys P-1 substrate decreases, and diverges sharply from its neutral and isosteric homolog, Met (Figure 28B). The similar and parallel upward trend seen with increasing positive charge for the Met and Glu P-1 substrates probably results from the fact that all the substrates are succinylated on their amino-terminal end, and thus carry a formal negative charge.

The trends observed in log kcat/Km are dominated by changes in the Km term (Figures 28C and 28D). As the pocket becomes more positively charged, the log 1/Km values converge for Glu and Gln P-1 substrates (Figure 28C), and diverge for Lys and Met P-1 substrates (Figure 28D). Although less

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pronounced effects are seen in log kcat, the effects of P-1 charge on log kcat parallel those seen in log 1/Km and become larger as the P-1 pocket becomes more positively charged. This may result from the fact that the transition-state is a tetrahedral anion, and a net positive charge in the enzyme may serve to provide some added stabilization to the transition-state.

The effect of the change in P-1 binding-site charge on substrate preference can be estimated from the differences in slopes between the charged and neutral isosteric P-1 substrates (Figure 28B). The average change in substrate preference (Δlog kcat/Km) between charged and neutral isosteric substrates increases roughly 10-fold as the complementary charge or the enzyme increases (Table XV). When comparing Glu versus Lys, this difference is 100-fold and the change in substrate preference appears predominantly in the Km term.

TABLE XV

Differential Effect on Binding Site Charge on log kcat/Km or (log 1/Km) for P-1 Substrates that Differ in Charge[a]			
Change in P-1 Binding Site Charge[b]	Δlog kcat/Km (Δlog 1/Km)		
	GluGln	MetLys	GluLys
-2 to -1	n.d.	1.2 (1.2)	n.d.
-1 to 0	0.7 (0.6)	1.3 (0.8)	2.1 (1.4)
0 to +1	1.5 (1.3)	0.5 (0.3)	2.0 (1.5)
Avg. change in log kcat/K_m or (log 1/Km) per unit charge change	1.1 (1.0)	1.0 (0.8)	2.1 (1.5)

[a] The difference in the slopes of curves were taken between the P-1 substrates over the charge interval given for log (kcat/Km) (Figure 28A, B) and (log 1/Km) (Figure 28C, D). Values represent the differential effect a charge change has in distinguishing the substrates that are compared.
[b] Charge in P-1 binding site is defined as the sum of charges from positions 156 and 166.

The free energy of electrostatic interactions in the structure and energetics of salt-bridge formation depends on the distance between the charges and the microscopic dielectric of the media. To dissect these structural and microenvironmental effects, the energies involved in specific salt-bridges were evaluated. In addition to the possible salt-bridges shown (Figures 29A and 29B), reasonable salt-bridges can be built between a Lys P-1 substrate and Asp at position 166, and between a Glu P-1 substrate and a Lys at position 166 (not shown). Although only one of these structures is confirmed by X-ray crystalography (Poulos, T.L., et al. (1976) J. Mol. Biol. 257 1097-1103), all models have favorable torsion angles (Sielecki, A.R., et al. (1979) J. Mol. Biol. 134, 781-804), and do not introduce unfavorable van der Waals contacts.

The change in charged P-1 substrate preference brought about by formation of the model salt-bridges above are shown in Table XVI.

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TABLE XVI

Effect of Salt Bridge Formation Between Enzyme and Substrate on P1 Substrate Preference[a]

Enzymes Compared[b] 1	Enzymes Compared[b] 2	Enzyme Position Changed	P-1 Substrates Compared	Substrate[d] Preference Δlog (kcat/Km) 1	Substrate[d] Preference Δlog (kcat/Km) 2	Change in Substrate Preferenc ΔΔlog (kcat/Km) (1-2)
Glu156/Asp166	Gln156/Asp166	156	LysMet	+0.30	-0.53	0.83
Glu156/Asn166	Gln156/Asn166	156	LysMet	-0.84	-2.04	1.20
Glu156/Gly166	Gln156/Gly166	156	LysMet	-0.47	-2.10	1.63
Glu156/Lsy-166	Gln156/Lys166	156	LysMet	-1.92	-2.74	0.82
				Ave ΔΔlog (kcat/Km)		1.10 ± 0.3
Glu156/Asp166	Glu156/Asn166	166	LysMet	+0.30	-0.84	1.14
Glu156/Glu166	Glu156/Glu166	166	LysMet	+0.62	-1.33	1.95
Gln156/Asp166	Gln156/Asn166	166	LysMet	-0.53	-2.04	1.51
Ser156/Asp166	Ser156/Asn166	166	LysMet	-0.43	-2.04	1.61
Glu156/Lys166	Glu156/Met166	166	GluGln	-0.63	-2.69	2/06
				Ave ΔΔlog (kcat/Km)		1.70 ± 0.3

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Footnotes to Table XVI:

(a) Molecular modeling shows it is possible to form a salt bridge betw en the indicated charged P-1 substrate and a complementary charge in the P-1 binding site of the enzyme at the indicated position changed.

(b) Enzymes compared have sterically similar amino acid substitutions that differ in charge at the indicated position.

(c) The P-1 substrates compared are structurally similar but differ in charge. The charged P-1 substrate is complementary to the charge change at the position indicated between enzymes 1 and 2.

(d) Date from Table XIV was used to compute the difference in log (kcat/Km) between the charged and the non-charged P-1 substrate (i.e., the substrate preference). The substrate preference is shown separately for enzyme 1 and 2.

(e) The difference in substrate preference between enzyme 1 (more highly charged) and enzyme 2 (more neutral) represents the rate change accompanying the electrostatic interaction.

The difference between catalytic efficiencies (i.e., Δlog kcat/Km) for the charged and neutral P-1 substrates (e.g., Lys minus Met or Glu minus Gln) give the substrate preference for each enzyme. The change in substrate preference (ΔΔlog kcat/Km) between the charged and more neutral enzyme homologs (e.g., Glu156/Gly166 minus Gln156(Q156)/Gly166) reflects the change in catalytic efficiency that may be attributed solely to electrostatic effects.

These results show that the average change in substrate preference is considerably greater when electrostatic substitutions are produced at position 166 (50-fold in kcat/Km) versus position 156 (12-fold in kcat/Km). From these ΔΔlog kcat/Km values, an average change in transition-state stabilization energy can be calculated of -1.5 and -2.4 kcal/mol for substitutions at positions 156 and 166, respectively. This should represent the stabilization energy contributed from a favorable electrostatic interaction for the binding of free enzyme and substrate to form the transition-state complex.

EXAMPLE 10

Substitutions at Position 217

Tyr217 has been substituted by all other 19 amino acids. Cassette mutagenesis as described in EPO publication No. 0130756 was used according to the protocol of Figure 22. The EcoRV restriction site was used for restriction-purification of pΔ217.

Since this position is involved in substrate binding, mutations here effect kinetic parameters of the enzyme. An example is the substitution of Leu for Tyr at position 217. For the substrate sAAPFpNa, this mutant has a kcat of 277 5' and a Km of 4.7×10^{-4} with a kcat/Km ratio of 6×10^{5}. This represents a 5.5-fold increase in kcat with a 3-fold increase in Km over the wild type enzyme.

In addition, replacement of Tyr217 by Lys, Arg, Phe or Leu results in mutant enzymes which are more stable at pHs of about 9-11 than the WT enzyme. Conversely, replacement of Tyr217 by Asp, Glu, Gly or Pro results in enzymes which are less stable at pHs of about 9-11 than the WT enzyme.

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EXAMPLE 11

Multiple Mutants Having Altered Thermal Stability

B. amyloliquefacien subtilisin does not contain any cysteine residues. Thus, any attempt to produce thermal stability by Cys cross-linkage required the substitution of more than one amino acid in subtilisin with Cys. The following subtilisin residues were multiply substituted with cysteine:

Thr22/Ser87

Ser24/Ser87

Mutagenesis of Ser24 to Cys was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

5'-pC-TAC-ACT-GGA-TG*C*-AAT-GTT-AAA-G-3'.

(Asterisks show the location of mismatches and the underlined sequence shows the position of the altered Sau3A site.) The B. amyloliquefaciens subtilisin gene on a 1.5 kb EcoRI-BAMHI fragment from pS4.5 was cloned into M13mp11 and single stranded DNA was isolated. This template (M13mp11SUBT) was double primed with the 5' phosphorylated M13 universal sequencing primer and the mutagenesis primer. Adelman, et al. (1983) DNA 2, 183-193. The heteroduplex was transfected into competent JM101 cells and plaques were probed for the mutant sequence (Zoller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500; Wallace, et al. (1981) Nucleic Acid Res. 9, 3647-3656) using a tetramethylammonium chloride hybridization protocol (Wood, et al. (1985) Proc. Natl. Acad. Sci. USA 82, 1585-1588). The Ser87 to Cys mutation was prepared in a similar fashion using a 5' phosphorylated primer having the sequence

5'-pGGC-GTT-GCG-CCA-*TGC-GCA-TCA-CT-3'.

(The asterisk indicates the position of the mismatch and the underlined sequence shows the position of a new MstI site.) The C24 and C87 mutations were obtained at a frequency of one and two percent, respectively. Mutant sequences were confirmed by dideoxy sequencing in M13.

Mutagenesis of Tyr21/Thr22 to A21/C22 was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

5'-pAC-TCT-CAA-GGC-*G*C*T-*TGT-GG*C-TCA-AAT-GTT-3'.

(The asterisks show mismatches to the wild type sequence and the underlined sequence shows the position of an altered Sau3A site.) Manipulations for heteroduplex synthesis were identical to those described for C24. Because direct cloning of the heteroduplex DNA fragment can yield increased frequencies of mutagenesis, the EcoRI-BamHI subtilisin fragment was purified and ligated into pBS42. E. coli MM 294 cells were transformed with the ligation mixture and plasmid DNA was purified from isolated transformants. Plasmid DNA was screened for the loss of the Sau3A site at codon 23 that was eliminated by the mutagenesis primer. Two out of 16 plasmid preparations had lost the wild type Sau3A site. The mutant sequence was confirmed by dideoxy sequencing in M13.

Double mutants, C22/C87 and C24/C87, were constructed by ligating fragments sharing a common ClaI site that separated the single parent cystine codons. Specifically, the 500 bp EcoRI-ClaI fragment containing the 5' portion of the subtilisin gene (including codons 22 and 24) was ligated with the 4.7 kb ClaI-EcoRI fragment that contained the 3' portion of the subtilisin gene (including codon 87) plus pBS42 vector sequence. E. coli MM 294 was transformed with ligation mixtures and plasmid DNA was purified from individual transformants. Double-cysteine plasmid constructions were identified by restriction site markers originating from the parent cysteine mutants (i.e., C22 and C24, Sau3A minus; Cys87, MstI plus). Plasmids from E. coli were transformed into B. subtilis BG2036. The thermal stability of these mutants as compared to wild type subtilisin are presented in Figure 30 and Tables XVII and XVIII.

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TABLE XVII

Effect of DTT on the Half-Time of Autolytic Inactivation of Wild-Type and Disulfide Mutants of Subtilisin*			
Enzyme	$t_{\frac{1}{2}}$		-DTT/+DTT
	-DDT	+DTT	
	min		
Wild-type	95	85	1.1
C22/C87	44	25	1.8
C24/C87	92	62	1.5

(*) Purified enzymes were either treated or not treated with 25mM DTT and dialyzed with or without 10mM DTT in 2mM $CaCl_2$, 50mM Tris (pH 7.5) for 14 hr. at 4°C. Enzyme concentrations were adjusted to 80µl aliquots were quenched on ice and assayed for residual activity. Half-times for autolytic inactivation were determined from semi-log plots of $\log_{10}$ (residual activity) versus time. These plots were linear for over 90% of the inactivation.

TABLE XVIII

Effect of Mutations in Subtilisin on the Half-Time of Autolytic Inactivation at 58°C*	
Enzyme	$t_{\frac{1}{2}}$
	min
Wild-type	120
C22	22
C24	120
C87	104
C22/C87	43
C24/C87	115

(*) Half-times for autolytic inactivation were determined for wild-type and mutant subtilisins as described in the legend to Table III. Unpurified and non-reduced enzymes were used directly from B. subtilis culture supernatants.

The disulfides introduced into subtilisin did not improve the autolytic stability of the mutant enzymes when compared to the wild-type enzyme. However, the disulfide bonds did provide a margin of autolytic stability when compared to their corresponding reduced double-cysteine enzyme. Inspection of a highly refined x-ray structure of wild-type B. amyloliquefaciens subtilisin reveals a hydrogen bond between Thr22 and Ser87. Because cysteine is a poor hydrogen donor or acceptor (Paul, I.C. (1974) in Chemistry of the -SH Group (Patai, S., ed.) pp. 111-149, Wiley Interscience, New York) weakening of 22/87 hydrogen bond may explain why the C22 and C87 single-cysteine mutant proteins are less autolytically stable than either C24 or wild-type (Table XVIII). The fact that C22 is less autolytically stable than C87 may be the result of the Tyr21A mutation (Table XVIII). Indeed, construction and analysis of Tyr21/C22 shows the mutant protein has an autolytic stability closer to that of C87. In summary, the C22 and C87 of single-cysteine mutations destabilize the protein toward autolysis, and disulfide bond formation increases the stability to a level less than or equal to that of wild-type enzyme.

EXAMPLE 12

Multiple Mutants Containing Substitutions at Position 222 and Position 166 or 169

Double mutants 166/222 and 169/222 were prepared by ligating together (1) the 2.3kb AcaII fragment from pS4.5 which contains the 5' portion of the subtilisin gene and vector sequences, (2) the 200bp AvaII fragment which contains the relevant 166 or 169 mutations from the respective 166 or 169 plasmids, and (3) the 2.2kb AvaII fragment which contains the relevant 222 mutation 3' and of the subtilisin genes and vector

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sequence from the respective p222 plasmid.

Although mutations at position 222 improve oxidation stability they also tend to increase the Km. An example is shown in Table XIX. In this case the A222 mutation was combined with the K166 mutation to give an enzyme with kcat and Km intermediate between the two parent enzymes.

TABLE XIX

	kcat	Km
WT	50	1.4×10^{-4}
A222	42	9.9×10^{-4}
K166	21	3.7×10^{-5}
K166/A222	29	2.0×10^{-4}
substrate sAAPFpNa		

EXAMPLE 13

Multiple Mutants Containing Substitutions at Positions 50, 156, 166, 217 and Combinations Thereof

The double mutant S156/A169 was prepared by ligation of two fragments, each containing one of the relevant mutations. The plasmid pS156 was cut with XmaI and treated with S1 nuclease to create a blunt end at codon 167. After removal of the nuclease by phenol/chloroform extraction and ethanol precipitation, the DNA was digested with BamHI and the approximately 4kb fragment containing the vector plus the 5' portion of the subtilisin gene through codon 167 was purified.

The pA169 plasmid was digested with KpnI and treated with DNA polymerase Klenow fragment plus 50 µM dNTPs to create a blunt end codon at codon 168. The Klenow was removed by phenol/chloroform extraction and ethanol precipitation. The DNA was digested with BamHI and the 590bp fragment including codon 168 through the carboxy terminus of the subtilisin gene was isolated. The two fragments were then ligated to give S156/A169.

Triple and quadruple mutants were prepared by ligating together (1) the 220bp PvuII/HaeII fragment containing the relevant 156, 166 and/or 169 mutations from the respective p156, p166 and/or p169 double of single mutant plasmid, (2) the 550bp HaeII/BamHI fragment containing the relevant 217 mutant from the respective p217 plasmid, and (3) the 3.9kb PvuII/BamHI fragment containing the F50 mutation and vector sequences.

The multiple mutant F50/S156/A169/L217, as well as B. amyloliquefaciens subtilisin, B. lichenformis subtilisin and the single mutant L217 were analyzed with the above synthetic polypeptides where the P-1 amino acid in the substrate was Lys, His, Ala, Gln, Tyr, Phe, Met and Leu. These results are shown in Figures 26 and 27.

These results show that the F50/S156/A169/L217 mutant has substrate specificity similar to that of the B. licheniformis enzyme and differs dramatically from the wild type enzyme. Although only data for the L217 mutant are shown, none of the single mutants (e.g., F50, S156 or A169) showed this effect. Although B. licheniformis differs in 88 residue positions from B. amyloliquefaciens, the combination of only these four mutations accounts for most of the differences in substrate specificity between the two enzymes.

EXAMPLE 14

Subtilisin Mutants Having Altered Alkaline Stability

A random mutagenesis technique was used to generate single and multiple mutations within the B. amyloliquefaciens subtilisin gene. Such mutants were screened for altered alkaline stability. Clones having increased (positive) alkaline stability and decreased (negative) alkaline stability were isolated and sequenced to identify the mutations within the subtilisin gene. Among the positive clones, the mutants V107 and R213 were identified. These single mutants were subsequently combined to produce the mutant V107/R213.

One of the negative clones (V50) from the random mutagenesis experiments resulted in a marked decrease in alkaline stability. Another mutant (P50) was analyzed for alkaline stability to determine the effect

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of a different substitution at position 50. The F50 mutant was found to have a greater alkaline stability than wild type subtilisin and when combined with the double mutant V107/R213 resulted in a mutant having an alkaline stability which reflected the aggregate of the alkaline stabilities for each of the individual mutants.

The single mutant R204 and double mutant C204/R213 were identified by alkaline screening after random cassette mutagenesis over the region from position 197 to 228. The C204/R213 mutant was thereafter modified to produce mutants containing the individual mutations C204 and R213 to determine the contribution of each of the individual mutations. Cassette mutagenesis using pooled oligonucleotides to substitute all amino acids at position 204, was utilized to determine which substitution at position 204 would maximize the increase in alkaline stability. The mutation from Lys213 to Arg was maintained constant for each of these substitutions at position 204.

A. Construction of pB0180, an E. coli-B. subtilis Shuttle Plasmid

The 2.9 kb EcoRI-BamHI fragment from pBR327 (Covarrubias, L., et al. (1981) Gene 13, 25-35) was ligated to the 3.7kb EcoRI-BamHI fragment of pBD64 (Gryczan, T., et al. (1980) J. Bacteriol., 141, 246-253) to give the recombinant plasmid pB0153. The unique EcoRI recognition sequence in pBD64 was eliminated by digestion with EcoRI followed by treatment with Klenow and deoxynucleotide triphosphates (Maniatis, T., et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Blunt end ligation and transformation yielded pB0154. The unique AvaI recognition sequence in pBO154 was eliminated in a similar manner to yield pBO171. pB0171 was digested with BamHI and PvuII and treated with Klenow and deoxynucleotide triphosphates to create blunt ends. The 6.4 kb fragment was purified, ligated and transformed into LE392 cells (Enquest, L.W., et al. (1977) J. Mol. Biol. 111, 97-120), to yield pB0172 which retains the unique BamHI site. To facilitate subcloning of subtilisin mutants, a unique and silent KpnI site starting at codon 166 was introduced into the subtilisin gene from pS4.5 (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925) by site-directed mutagenesis. The KpnI+ plasmid was digested with EcoRI and treated with Klenow and deoxynucleotide triphosphates to create a blunt end. The Klenow was inactivated by heating for 20 min at 68°C, and the DNA was digested with BamHI. The 1.5 kb blunt EcoRI-BamHI fragment containing the entire subtilisin was ligated with the 5.8 kb NruI-BamHI from pB0172 to yield pBO180. The ligation of the blunt NruI end to the blunt EcoRI end recreated an EcoRI site. Proceeding clockwise around pB0180 from the EcoRI site at the 5' end of the subtilisin gene is the unique BamHI site at the 3' end of the subtilisin gene, the chloramphenicol and neomycin resistance genes and UB110 gram positive replication origin derived from pBD64, the ampicillin resistance gene and gram negative replication origin derived from pBR327.

B. Construction of Random Mutagenesis Library

The 1.5 kb EcoRI-BamHI fragment containing the B. amyloliquefaciens subtilisin gene (Wells et al., 1983) from pB0180 was cloned into M13mp11 to give M13mp11 SUBT essentially as previously described (Wells, J.A., et al. (1986) J. Biol. Chem., 261,6564-6570). Deoxyuridine containing template DNA was prepared according to Kunkel (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA, 82 488-492). Uridine containing template DNA (Kunkel, 1985) was purified by CsCl density gradients (Maniatis, T. et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A primer (AvaI$^-$) having the sequence

5'GAAAAAAGACCC*TAGCGTCGCTTA

ending at codon -11, was used to alter the unique AvaI recognition sequence within the subtilisin gene. (The asterisk denotes the mismatches from the wild-type sequence and underlined is the altered AvaI site.)

The 5' phosphorylated AvaI primer (~320 pmol) and ~40 pmol (~120μg) of uridine containing M13mp11 SUBT template in 1.88 ml of 53 mM NaCl, 7.4 mM MgCl2 and 7.4 mM Tris.HCl (pH 7.5) were annealed by heating to 90°C for 2 min. and cooling 15 min at 24°C (Fig. 31). Primer extension at 24°C was initiated by addition of 100μL containing 1 mM in all four deoxynucleotide triphosphates, and 20μl Klenow fragment (5 units/l). The extension reaction was stopped every 15 seconds over ten min by addition of 10μl 0.25 M EDTA (pH 8) to 50μl aliquots of the reaction mixture. Samples were pooled, phenol chlorophorm extracted and DNA was precipitated twice by addition of 2.5 vol 100% ethanol, and washed twice with 70% ethanol.

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The pellet was dried, and redissolved in 0.4 ml 1 mM EDTA, 10 mM Tris (pH 8).

Misincorporation of α-thiodeoxynucleotides onto the 3' ends of the pool of randomly terminated template was carried out by incubating four 0.2 ml solutions each containing one-fourth of the randomly terminated template mixture (~20µg), 0.25 mM of a given α-thiodeoxynucleotide triphosphate, 100 units AMV polymerase, 50 mM KCL, 10 mM $MgCl_2$, 0.4 mM dithiothreitol, and 50 mM Tris (pH 8.3) (Champoux, J.J. (1984) Genetics, 2, 454-464). After incubation at 37 • C for 90 minutes, misincorporation reactions were sealed by incubation for five minutes at 37 • C with 50 mM all four deoxynucleotide triphosphates (pH 8), and 50 units AMV polymerase. Reactions were stopped by addition of 25 mM EDTA (final), and heated at 68 • C for ten min to inactivate AMV polymerase. After ethanol precipitation and resuspension, synthesis of closed circular heteroduplexes was carried out for two days at 14 • C under the same conditions used for the timed extension reactions above, except the reactions also contained 1000 units T4 DNA ligase, 0.5 mM ATP and 1 mM β-mercaptoethanol. Simultaneous restriction of each heteroduplex pool with KpnI, BamHI, and EcoRI confirmed that the extension reactions were nearly quantitative. Heteroduplex DNA in each reaction mixture was methylated by incubation with 80µM S-adenosylmethionine and 150 units dam methylase for 1 hour at 37 • C. Methylation reactions were stopped by heating at 68 • C for 15 min.

One-half of each of the four methylated heteroduplex reactions were transformed into 2.5 ml competent E. coli JM101 (Messing, J. (1979) Recombinant DNA Tech. Bull., 2, 43-48). The number of independent transformants from each of the four transformations ranged from 0.4-2.0 x 10^5. After growing out phage pools, RF DNA from each of the four transformations was isolated and purified by centrifugation through CsCl density gradients. Approximately 2µg of RF DNA from each of the four pools was digested with EcoRI, BamHI and AvaI. The 1.5 kb EcoRI-BamHI fragment (i.e., AvaI resistant) was purified on low gel temperature agarose and ligated into the 5.5 kb EcoRI-BamHI vector fragment of pB0180. The total number of independent transformants from each α-thiodeoxynucleotide misincorporation plasmid library ranged from 1.2-2.4 x 10^4. The pool of plasmids from each of the four transformations was grown out in 200 ml LB media containing 12.5µg/ml cmp and plasmid DNA was purified by centrifugation through CsCl density gradients.

C. Expression and Screening of Subtilisin Point Mutants

Plasmid DNA from each of the four misincorporation pools was transformed (Anagnostopoulos, C., et al. (1967), J. Bacteriol., 81, 741-746) into BG2036. For each transformation, 5µg of DNA produced approximately 2.5 x 10^5 independent BG2036 transformants, and liquid culture aliquots from the four libraries were stored in 10% glycerol at 70 • C. Thawed aliquots of frozen cultures were plated on LB/5µg/ml cmp/1.6% skim milk plates (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925), and fresh colonies were arrayed onto 96-well microtiter plates containing 150 l per well LB media plus 12.5µg/ml cmp. After 1 h at room temperature, a replica was stamped (using a matched 96 prong stamp) onto a 132 mm BA 85 nitrocellulose filter (Schleicher and Scheull) which was layered on a 140 mm diameter LB/cmp/skim milk plate. Cells were grown about 16 h at 30 • C until halos of proteolysis were roughly 5-7 mm in diameter and filters were transferred directly to a freshly prepared agar plate at 37 • C containing only 1.6% skim milk and 50 mM sodium phosphate pH 11.5. Filters were incubated on plates for 3-6 h at 37 • C to produce halos of about 5 mm for wild-type subtilisin and were discarded. The plates were stained for 10 min at 24 • C with Coomassie blue solution (0.25% Coomassie blue (R-250) 25% ethanol) and destained with 25% ethanol, 10% acetic acid for 20 min. Zones of proteolysis appeared as blue halos on a white background on the underside of the plate and were compared to the original growth plate that was similarly stained and destained as a control. Clones were considered positive that produced proportionately larger zones of proteolysis on the high pH plates relative to the original growth plate. Negative clones gave smaller halos under alkaline conditions. Positive and negative clones were restreaked to colony purify and screened again in triplicate to confirm alkaline pH results.

D. Identification and Analysis of Mutant Subtilisins

Plasmid DNA from 5 ml overnight cultures of more alkaline active B.subtilis clones was prepared according to Birnboim and Doly (Birnboim, H.C., et al. (1979) Nucleic Acid Res. 7, 1513) except that incubation with 2 mg/ml lysozyme proceeded for 5 min at 37 • C to ensure cell lysis and an additional phenol/$CHCl_3$ extraction was employed to remove contaminants. The 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was ligated into M13mp11 and template DNA was prepared for DNA sequencing (Messing, J., et al. (1982) Gene, 19 269-276). Three DNA sequencing primers ending at codon 26, +95, and +155 were synthesized to match the subtilisin coding sequence. For preliminary sequence

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identification a single track of DNA sequence, corresponding to the dNTPaS misincorporation library from which the mutant came, was applied over the entire mature protein coding sequence (i.e., a single dideoxyguanosine sequence track was applied to identify a mutant from the dGTPas library). A complete four track of DNA sequence was performed 200 bp over the site of mutagenesis to confirm and identify the mutant sequence (Sanger, F., et al., (1980) J. Mol. Biol., 143, 161-178). Confirmed positive and negative bacilli clones were cultured in LB media containing 12.5µg/mL cmp and purified from culture supernatants as previously described (Estell, D.A., et al. (1985) J. Biol. Chem., 260, 6518-6521). Enzymes were greater than 98% pure as analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, U.K. (1970), Nature, 227, 680-685), and protein concentrations were calculated from the absorbance at 280 nm,

$$\epsilon_{280}^{0.1\%} = 1.17$$

(Maturbara, H., et al. (1965), J. Biol. Chem, 240, 1125-1130).

Enzyme activity was measured with 200µg/mL succinyl-L-AlaL-AlaL-ProL-Phep-nitroanilide (Sigma) in 0.1M Tris pH 8.6 or 0.1 M CAPS pH 10.8 at 25°C. Specific activity (µ moles product/min-mg) was calculated from the change in absorbance at 410 nm from production of p-nitroaniline with time per mg of enzyme (E410 = 8,480 M-lcm-l; Del Mar, E.G., et al. (1979), Anal. Biochem., 99, 316-320). Alkaline autolytic stability studies were performed on purified enzymes (200µg/mL) in 0.1 M potassium phosphate (pH 12.0) at 37°C. At various times aliquots were assayed for residual enzyme activity (Wells, J.A., et al. (1986) J. Biol. Chem., 261, 6564-6570).

E. Results

1. Optimization and analysis of mutagenesis frequency

A set of primer-template molecules that were randomly 3'-terminated over the subtilisin gene (Fig. 31) was produced by variable extension from a fixed 5'-primer (The primer mutated a unique AvaI site at codon 11 in the subtilisin gene). This was achieved by stopping polymerase reactions with EDTA after various times of extension. The extent and distribution of duplex formation over the 1 kb subtilisin gene fragment was assessed by multiple restriction digestion (not shown). For example, production of new HinfI fragments identified when polymerase extension had proceeded past Ile110, Leu233, and Asp259 in the subtilisin gene.

Misincorporation of each dNTPαs at randomly terminated 3' ends by AMV reverse transcriptase (Zakour, R.A., et al. (1982), Nature, 295, 708-710; Zakour, R.A., et al. (1984), Nucleic Acids Res., 12, 6615-6628) used conditions previously described (Champoux, J.J., (1984), Genetics, 2, 454-464). The efficiency of each misincorporation reaction was estimated to be greater than 80% by the addition of each dNTPαs to the AvaI restriction primer, and analysis by polyacrylamide gel electrophoresis. Misincorporations were sealed by polymerization with all four dNTP's and closed circular DNA was produced by reaction with DNA ligase.

Several manipulations were employed to maximize the yield of the mutant sequences in the heteroduplex. These included the use of a deoxyuridine containing template (Kunkel, T.A. (1985), Proc. Natl. Acad. Sci. USA, 82 488-492; Pukkila, P.J. et al. (1983), Genetics, 104, 571-582), in vitro methylation of the mutagenic strand (Kramer, W. et al. (1982) Nucleic Acids Res., 10 6475-6485), and the use of AvaI restriction-selection against the wild-type template strand which contained a unique AvaI site. The separate contribution of each of these enrichment procedures to the final mutagenesis frequency was not determined, except that prior to AvaI restriction-selection roughly one-third of the segregated clones in each of the four pools still retained a wild-type AvaI site within the subtilisin gene. After AvaI restriction-selection greater than 98% of the plasmids lacked the wild-type AvaI site.

The 1.5 kb EcoRI-BamHI subtilisin gene fragment that was resistant to AvaI restriction digestion, from each of the four CsCl purified M13 RF pools was isolated on low melting agarose. The fragment was ligated in situ from the agarose with a similarly cut E. coli-B. subtilis shuttle vector, pB0180, and transformed directly into E coli LE392. Such direct ligation and transformation of DNA isolated from agarose avoided loses and allowed large numbers of recombinants to be obtained (>100,000 per µg equivalent of input M13 pool).

The frequency of mutagenesis for each of the four dNTPαs misincorporation reactions was estimated from the frequency that unique restriction sites were eliminated (Table XX). The unique restriction sites

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chosen for this analysis, ClaI, PvuII, and KpnI, were distributed over the subtilisin gene starting at codons 35, 104, and 166, respectively. As a control, the mutagenesis frequency was determined at the PstI site located in the β lactamase gene which was outside the window of mutagenesis. Because the absolute mutagenesis frequency was close to the percentage of undigested plasmid DNA, two rounds of restriction-selection were necessary to reduce the background of surviving uncut wild-type plasmid DNA below the mutant plasmid (Table XX). The background of surviving plasmid from wild-type DNA probably represents the sum total of spontaneous mutations, uncut wild-type plasmid, plus the efficiency with which linear DNA can transform E. coli. Subtracting the frequency for unmutagenized DNA (background) from the frequency for mutant DNA, and normalizing for the window of mutagenesis sampled by a given restriction analysis (4-6 bp) provides an estimate of the mutagenesis efficiency over the entire coding sequence (~1000 bp).

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TABLE XX

α-thiol dNTP misincorporated[b]	Restriction Site Selection	% resistant clones[c] 1st round	2nd round	Total	% resistant clones over Background[d]	% mutants per 1000bp[e]
None	PstI	0.32	0.7	0.002	0	-
G	PstI	0.33	1.0	0.003	0.001	0.2
T	PstI	0.32	<0.5	<0.002	0	0
C	PstI	0.43	3.0	0.013	0.011	3
None	ClaI	0.28	5	0.014	0	-
G	ClaI	2.26	85	1.92	1.91	380
T	ClaI	0.48	31	0.15	0.14	35
C	ClaI	0.55	15	0.08	0.066	17
None	PvuII	0.08	29	0.023	0	-
G	PvuII	0.41	90	0.37	0.35	88
T	PvuII	0.10	67	0.067	0.044	9
C	PvuII	0.76	53	0.40	0.38	95
None	KpnI	0.41	3	0.012	0	-
G	KpnI	0.98	35	0.34	0.33	83
T	KpnI	0.36	15	0.054	0.042	8
C	KpnI	1.47	26	0.38	0.37	93

(a) Mutagenesis frequency is estimated from the frequency for obtaining mutations that alter unique restriction sites within the mutagenized subtilisin gene (i.e., ClaI, PvuII, or KpnI) compared to mutation frequencies of the PstI site, that is outside the window of mutagenesis.

(b) Plasmid DNA was from wild-type (none) or mutagenized by dNTPαs misincorporation as described.

(c) Percentage of resistant clones was calculated from the fraction of clones obtained after three fold or greater over-digestion of the plasmid with the indicated restriction enzyme compared to a

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non-digested control. Restriction-resistant plasmid DNA from the first round was subjected to a second round of restriction-selection. The total represents the product of the fractions of resistant clones obtained from both rounds of selection and gives percentage of restriction-site mutant clones in the original starting pool. Frequencies were derived from counting at least 20 colonies and usually greater than 100.

(d) Percent resistant clones was calculated by subtracting the percentage of restriction-resistant clones obtained for wild-type DNA (i.e., none) from that obtained for mutant DNA.

(e) This extrapolates from the frequency of mutation over each restriction site to the entire subtilisin gene (~1 kb). This has been normalized to the number of possible bases (4-6 bp) within each restriction site that can be mutagenized by a given misincorporation event.

From this analysis, the average percentage of subtilisin genes containing mutations that result from dGTPαs, dCTPαs, or dTTPαs misincorporation was estimated to be 90, 70, and 20 percent, respectively. These high mutagenesis frequencies were generally quite variable depending upon the dNTPαs and misincorporation efficiencies at this site. Misincorporation efficiency has been reported to be both dependent on the kind of mismatch, and the context of primer (Champoux, J.J., (1984); Skinner, J.A., et al. (1986) Nucleic Acids Res., 14, 6945-6964). Biased misincorporation efficiency of dGTPαs and dCTPαs over dTTPαs has been previously observed (Shortle, D., et al. (1985), Genetics, 110, 539-555). Unlike the dGTPαs, dCTPαs, and dTTPαs libraries the efficiency of mutagenesis for the dATPαs misincorporation library could not be accurately assessed because 90% of the restriction-resistant plasmids analyzed simply lacked the subtilisin gene insert. This problem probably arose from self-ligation of the vector when the dATPαs mutagenized subtilisin gene was subcloned from M13 into pB0180. Correcting for the vector background, we estimate the mutagenesis frequency around 20 percent in the dATPαs misincorporation library. In a separate experiment (not shown), the mutagenesis efficiencies for dGTPαs and dTTPαs misincorporation were estimated to be around 50 and 30 percent, respectively, based on the frequency of reversion of an inactivating mutation at codon 169.

The location and identity of each mutation was determined by a single track of DNA sequencing corresponding to the misincorporated αthiodeoxynucleotide over the entire gene followed by a complete four track of DNA sequencing focused over the site of mutation. Of 14 mutants identified, the distribution was similar to that reported by Shortle and Lin (1985) except we did not observe nucleotide insertion or deletion mutations. The proportion of AG mutations was highest in the G misincorporation library, and some unexpected point mutations appeared in the dTTPαs and dCTPαs libraries.

2. Screening and Identification of Alkaline Stability Mutants of Subtilisin

It is possible to screen colonies producing subtilisin by halos of casein digestion (Wells, J.A. et al. (1983) Nucleic Acids Res., 11, 7911-7925). However, two problems were posed by screening colonies under high alkaline conditions (>pH 11). First, B. subtilis will not grow at high pH, and we have been unable to transform an alkylophilic strain of bacillus. This problem was overcome by adopting a replica plating strategy in which colonies were grown on filters at neutral pH to produce subtilisin and filters subsequently transferred to casein plates at pH 11.5 to assay subtilisin activity. However, at pH 11.5 the casein micells no longer formed a turbid background and thus prevented a clear observation of proteolysis halos. The problem was overcome by briefly staining the plate with Coomassie blue to amplify proteolysis zones and acidifying the plates to develop casein micell turbidity. By comparison of the halo size produced on the reference growth plate (pH 7) to the high pH plate (pH 11.5), it was possible to identify mutant subtilisins that had increased (positives) or decreased (negatives) stability under alkaline conditions.

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Roughly 1000 colonies were screened from each of the four misincorporation libraries. The percentage of colonies showing a differential loss of activity at pH 11.5 versus pH 7 represented 1.4, 1.8, 1.4, and 0.6% of the total colonies screened from the thiol dGTPαs, dATPαs, dTTPαs, and dCTPαs libraries, respectively. Several of these negative clones were sequenced and all were found to contain a single base change as expected from the misincorporation library from which they came. Negative mutants included A36, E170 and V50. Two positive mutants were identified as V107 and R213. The ratio of negatives to positives was roughly 50:1.

3. Stability and Activity of Subtilisin Mutants at Alkaline pH

Subtilisin mutants were purified and their autolytic stabilities were measured by the time course of inactivation at pH 12.0 (Figs. 32 and 33). Positive mutants identified from the screen (i.e., V107 and R213) were more resistant to alkaline induced autolytic inactivation compared to wild-type; negative mutants (i.e., E170 and V50) were less resistant. We had advantageously produced another mutant at position 50 (F50) by site-directed mutagenesis. This mutant was more stable than wild-type enzyme to alkaline autolytic inactivation (Fig. 33) At the termination of the autolysis study, SDS-PAGE analysis confirmed that each subtilisin variant had autolyzed to an extent consistent with the remaining enzyme activity.

The stabilizing effects of V107, R213, and F50 are cumulative. See Table XXI. The double mutant, V107/R213 (made by subcloning the 920 bp EcoRI-KpnI fragment of pB0180V107 into the 6.6 kb EcoRI-KpnI fragment of pB0180R213), is more stable than either single mutant. The triple mutant, F50/V107/R213 (made by subcloning the 735 bp EcoRI-PvuII fragment of pF50 (Example 2) into the 6.8 kb EcoRI-PvuII fragment of pB0180/V107, is more stable than the double mutant V107/R213 or F50. The inactivation curves show a biphasic character that becomes more pronounced the more stable the mutant analyzed. This may result from some destablizing chemical modification(s) (eg., deamidation) during the autolysis study and/or reduced stabilization caused by complete digestion of larger autolysis peptides. These alkaline autolysis studies have been repeated on separately purified enzyme batches with essentially the same results. Rates of autolysis should depend both on the conformational stability as well as the specific activity of the subtilisin variant (Wells, J.A., et al. (1986), J. Biol. Chem., 261, 6564-6570). It was therefore possible that the decreases in autolytic inactivation rates may result from decreases in specific activity of the more stable mutant under alkaline conditions. In general the opposite appears to be the case. The more stable mutants, if anything, have a relatively higher specific activity than wild-type under alkaline conditions and the less stable mutants have a relatively lower specific activity. These subtle effects on specific activity for V107/R213 and F50/V107/R213 are cumulative at both pH 8.6 and 10.8. The changes in specific activity may reflect slight differences in substrate specificity, however, it is noteworthy that only positions 170 and 107 are within 6A of a bound model substrate (Robertus, J.D., et al. (1972), Biochemistry 11, 2438-2449).

TABLE XXI

Relationship between relative specific acitivity at pH 8.6 or 10.8 and alkaline autolytic stability

Enzyme	Relative specific activity		Alkaline autolysis half-time (min)b
	pH 8.6	pH 10.8	
Wild-type	100±1	100±3	86
Q170	46±1	28±2	13
V107	126±3	99±5	102
R213	97±1	102±1	115
V107/R213	116±2	106±3	130
V50	68±4	61±1	58
F50	123±3	157±7	131
F50/V107/R213	126±2	152±3	168

(a) Relative specific activity was the average from triplicate activity determinations divided by the wild-type value at the same pH. The average specific activity of wild-type enzyme at pH 8.6 and 10.8 was 70µmoles/min-mg and 37µmoles/min-mg, respectively.

(b) Time to reach 50% activity was taken from Figs. 32 and 33.

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F. Random Cassette Mutagenesis of Residues 197 through 228

Plasmid pΔ222 (Wells, et al. (1985) Gene 34, 315-323) was digested with PstI and BamHI and the 0.4 kb PstI/BamHI fragment (fragment 1, see Fig. 34) purified from a polyacrylamide gel by electroelution.

The 1.5 kb EcoRI/BamHI fragment from pS4.5 was cloned into M13mp9. Site directed mutagenesis was performed to create the A197 mutant and simultaneously insert a silent SstI site over codons 195-196. The mutant EcoRI/BamHI fragment was cloned back into pBS42. The pA197 plasmid was digested with BamHI and SstI and the 5.3 kb BamHI/SstI fragment (fragment 2) was purified from low melting agarose.

Complimentary oligonucleotides were synthesized to span the region from SstI (codons 195-196) to PstI (codons 228-230). These oligodeoxynucleotides were designed to (1) restore codon 197 to the wild type, (2) re-create a silent KpnI site present in pΔ222 at codons 219-220, (3) create a silent SmaI site over codons 210-211, and (4) eliminate the PstI site over codons 228-230 (see Fig. 35). Oligodeoxynucleotides were synthesized with 2% contaminating nucleotides at each cycle of synthesis, e.g., dATP reagent was spiked with 2% dCTP, 2% dGTP, and 2% dTTP. For 97-mers, this 2% poisoning should give the following percentages of non-mutant, single mutants and double or higher mutants per strand with two or more misincorporations per complimentary strand: 14% non-mutant, 28% single mutant, and 57% with ≥2 mutations, according to the general formula

$$f = \frac{\mu^n}{n!} e^{-\mu} .$$

where μ is the average number of mutations and n is a number class of mutations and f is the fraction of the total having that number of mutations. Complimentary oligodeoxynucleotide pools were phosphorylated and annealed (fragment 3) and then ligated at 2-fold molar excess over fragments 1 and 2 in a three-way ligation.

E. coli MM294 was transformed with the ligation reaction, the transformation pool grown up over night and the pooled plasmid DNA was isolated. This pool represented 3.4×10^4 independent transformants. This plasmid pool was digested with PstI and then used to retransform E. coli. A second plasmid pool was prepared and used to transform B. subtilis (BG2036). Approximately 40% of the BG2036 transformants actively expressed subtilisin as judged by halo-clearing on casein plates. Several of the non-expressing transformants were sequenced and found to have insertions or deletions in the synthetic cassettes. Expressing BG2036 mutants were arrayed in microtiter dishes with 150µl of LB/12.5µg/mL chloramphenicol (cmp) per well, incubated at 37 °C for 3-4 hours and then stamped in duplicate onto nitrocellulose filters laid on LB 1.5% skim milk/5µg/mL cmp plates and incubated overnight at 33 °C (until halos were approximately 4-8 mm in diameter). Filters were then lifted to stacks of filter paper saturated with 1 x Tide commercial grade detergent, 50 mM Na_2CO_3, pH 11.5 and incubated at 65 °C for 90 min. Overnight growth plates were Commassie stained and destained to establish basal levels of expression. After this treatment, filters were returned to pH7/skim milk/20µg/mL tetracycline plates and incubated at 37 °C for 4 hours to overnight.

Mutants identified by the high pH stability screen to be more alkaline stable were purified and analyzed for autolytic stability at high pH or high temperature. The double mutant C204/R213 was more stable than wild type at either high pH or high temperature (Table XXII).

This mutant was dissected into single mutant parents (C204 and R213) by cutting at the unique SmaI restriction site (Fig. 35) and either ligating wild type sequence 3' to the SmaI site to create the single C204 mutant or ligating wild type sequence 5' to the SmaI site to create the single R213 mutant. Of the two single parents, C204 was nearly as alkaline stable as the parent double mutant (C04/R213) and slightly more thermally stable. See Table XXII. The R213 mutant was only slightly more stable than wild type under both conditions (not shown).

Another mutant identified from the screen of the 197 to 228 random cassette mutagenesis was R204. This mutant was more stable than wild type at both high pH and high temperature but less stable than C204.

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TABLE XXII

Stability of subtilisin variants

Purified enzymes (200μg/mL) were incubated in 0.1M phosphate, pH 12 at 30°C for alkaline autolysis, or in 2mM $CaCl_2$, 50mM MOPS, pH 7.0 at 62°C for thermal autolysis. At various times samples were assayed for residual enzyme activity. Inactivations were roughly pseudo-first order, and t 1/2 gives the time it took to reach 50% of the starting activity in two separate experiments.

	t 1/2 (alkaline autolysis)		t 1/2 (thermal autolysis)	
Subtilisin variant	Exp. #1	Exp. #2	Exp. #1	Exp. #2
wild type	30	25	20	23
F50/V107/R213	49	41	18	23
R204	35	32	24	27
C204	43	46	38	40
C204/R213	50	52	32	36
L204/R213	32	30	20	21

G. Random Mutagenesis at Codon 204

Based on the above results, codon 204 was targeted for random mutagenesis. Mutagenic DNA cassettes (for codon at 204) all contained a fixed R213 mutation which was found to slightly augment the stability of the C204 mutant.

Plasmid DNA encoding the subtilisin mutant C204/R213 was digested with SstI and EcoRI and a 1.0 kb EcoRI/SstI fragment was isolated by electro-elution from polyacrylamide gel (fragment 1, see Fig. 35).

C204/R213 was also digested with SmaI and EcoRI and the large 4.7 kb fragment, including vector sequences and the 3' portion of coding region, was isolated from low melting agarose (fragment 2, see Fig. 36).

Fragments 1 and 2 were combined in four separate three-way ligations with heterophosphorylated fragments 3 (see Figs. 36 and 37). This heterophosphorylation of synthetic duplexes should preferentially drive the phosphorylated strand into the plasmid ligation product. Four plasmid pools, corresponding to the four ligations, were restricted with SmaI in order to linearize any single cut C204/R213 present from fragment 2 isolation, thus reducing the background of C204/R213. E. coli was then re-transformed with

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SmaI-restricted plasmid pools to yield a second set of plasmid pools which are essentially free of C204/R213 and any non-segregated heterduplex material.

These second enriched plasmid pools were then used to transform B. subtilis (BG2036) and the resulting four mutant pools were screened for clones expressing subtilisin resistant to high pH/temperature inactivation. Mutants found positive by such a screen were further characterized and identified by sequencing.

The mutant L204/R213 was found to be slightly more stable than the wild type subtilisin. See Table XXII.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

Claims

1. A subtilisin mutant derived by the substitution of at least one amino acid residue of a precursor subtilisin with a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterised by the substitution at one or more of Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of Bacillus amyloliquefaciens subtilisin and equivalent amino acid residues in other precursor subtilisins.

2. A subtilisin mutant having an amino acid sequence derived from the amino acid sequence of a precursor subtilisin by the substitution of more than one amino acid residue of said amino acid sequence of said precursor subtilisin by a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterized by substitutions at more than one of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of Bacillus amyloliquefaciens subtilisin and equivalent amino acid residues in other precursor subtilisins, with the proviso that when substitution is made at any residue in the group Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156 Gly166, Gly169, Phe189, Tyr217 and Met222 a substitution is also made at at least one specified position not of that group.

3. The mutant of claim 2 wherein said combinations are selected from Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 and Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

4. A subtilisin mutant derived by the deletion of one or more amino acid residues in a precursor subtilisin equivalent to 161-164 in B. amyloliquefaciens subtilisin, said deletion being made alone or in combination with substitutions in the amino acid sequence of the precursor subtilisin, and producing at least one property which is different from the same property of the precursor subtilisin.

5. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Leu + 126 of B. amyloliquefaciens subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.

6. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Asp + 99 in B. amyloliquefaciens subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.

7. A DNA sequence encoding the mutant of any one of the preceding claims.

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8. An expression vector containing the mutant DNA sequence of claim 7.

9. A host cell transformed with the expression vector or claim 8.

Patentansprüche

1. Subtilisinmutante, die durch Substitution zumindest eines Aminosäurerests eines Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft aufweist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch die Substitution an einem oder mehreren von Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von Bacillus amyloliquefaciens-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen.

2. Subtilisinmutante mit einer Aminosäuresequenz, die aus der Aminosäuresequenz eines Vorläufer-Subtilisins durch Substitution mehr als eines Aminosäurerests der Aminosäuresequenz des Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft auWeist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch Substitutionen an mehr als einem von Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von Bacillus amyloliquefaciens-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen, mit der Maßgabe, daß bei einer Substitution an irgendeinem Rest in der Gruppe Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 und Met222 eine Substitution auch an zumindest einer bestimmten Position durchgeführt wird, die nicht dieser Gruppe angehört.

3. Mutante nach Anspruch 2, worin die Kombinationen aus Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Tyr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 und Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217 ausgewählt sind.

4. Subtilisinmutante, die durch Löschung eines oder mehrerer Aminosäurereste in einem Vorläufer-Subtilisin, das 161-164 in B. amyloliquefaciens-Subtilisin äquivalent ist, hergeleitet ist, wobei die Löschung entweder alleine oder in Kombination mit Substitutionen in der Aminosäuresequenz des Vorläufer-Subtilisins erfolgt, und zumindest eine Eigenschaft ergibt, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet.

5. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufersubtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Leu + 126 von B. amyloliquefaciens-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.

6. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufersubtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Asp +99 im B. amyloliquefaciens-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.

7. DNA-Sequenz, die für die Mutante nach einem der vorhergehenden Ansprüche kodiert.

8. Expressionsvektor, der die Mutanten-DNA-Sequenz von Anspruch 7 enthält.

9. Wirtszelle, die mit dem Expressionsvektor von Anspruch 8 transformiert ist.

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Revendications

1. Mutant de subtilisine dérivé par la substitution d'au moins un résidu d'acide aminé d'une subtilisine précurseur et par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par la substitution à un ou plusieurs de Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilise de Bacillus amyloliquefaciens et les résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs.

2. Mutant de subtilisine ayant une séquence d'acides aminés dérivée de la séquence d'acides aminés d'une subtilisine précurseur par la substitution de plus d'un résidu d'acide aminé de ladite séquence d'acides aminés de ladite subtilisine précurseur par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par des substitutions à plus d'un de Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilisine de Bacillus amyloliquefaciens et des résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs, à condition que quand la substitution est effectuée à tout résidu dans le groupe formé de Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 et Met222, une substitution soit également effectuée en au moins une position spécifiée ne faisant pas partie de ce groupe.

3. Mutant de la revendication 2 où lesdites associations sont choisies parmi Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 et Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

4. Mutant de subtilisine dérivé par la délétion d'un ou plusieurs résidus d'acides aminés dans une subtilisine précurseur équivalente à 161-164 dans la subtilisine de B. amyloliquefaciens, ladite délétion étant effectuée seule ou en association avec des substitutions dans la séquence d'acides aminés de la subtilisine précurseur et la production d'au moins une propriété qui est différente de la même propriété de la subtilisine précurseur.

5. Mutant de subtilisine ayant une spécificité modifiée du substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Leu + 126 de la subtilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.

6. Mutant de subtilisine ayant une spécificité modifiée de substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Asp + 99 dans la substilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.

7. Séquence d'ADN codant le mutant selon l'une quelconque des revendications précédentes.

8. Vecteur d'expression contenant la séquence d'ADN du mutant de la revendication 7.

9. Cellule hôte transformée par le vecteur d'expression de la revendication.8 .

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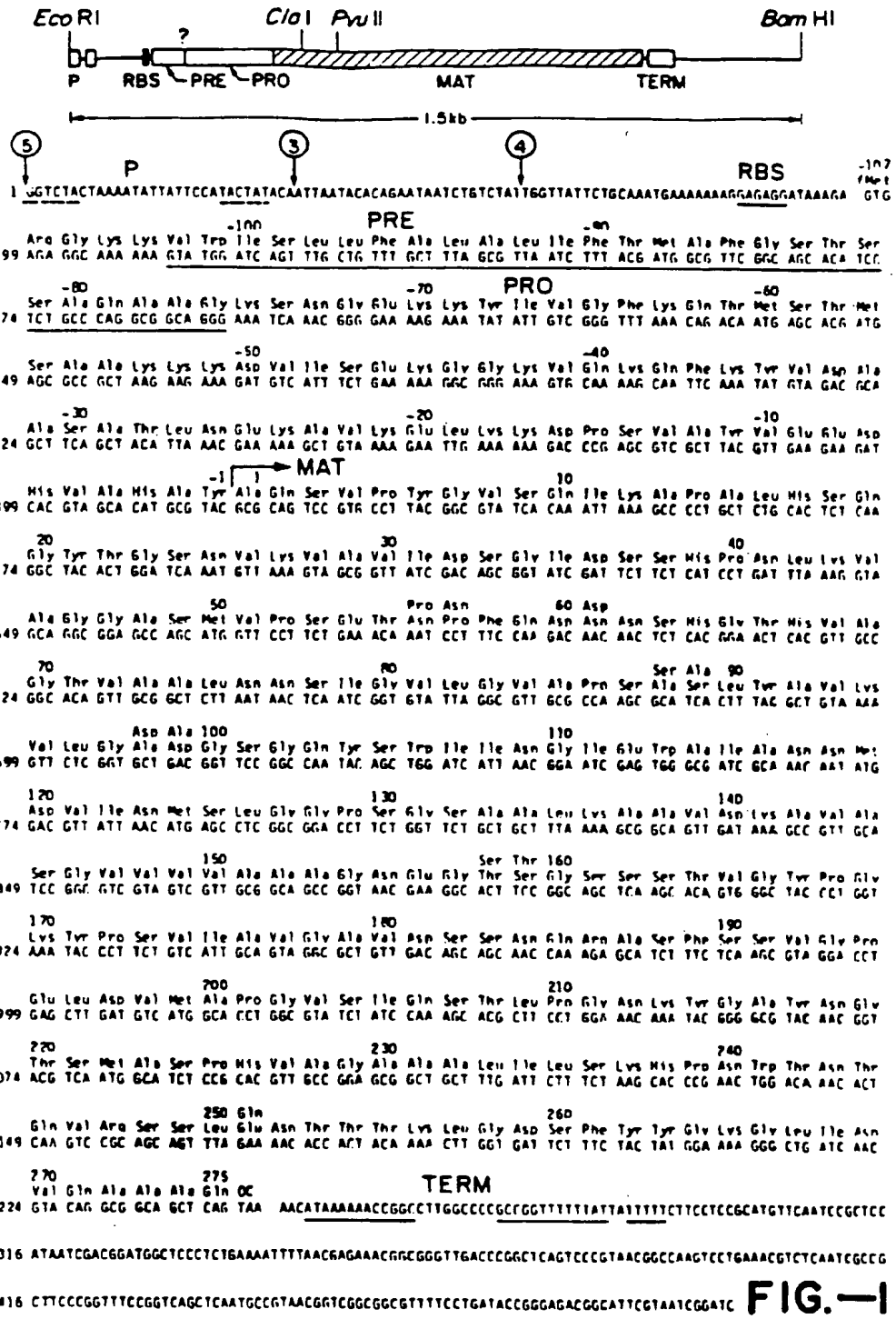

FIG.—1

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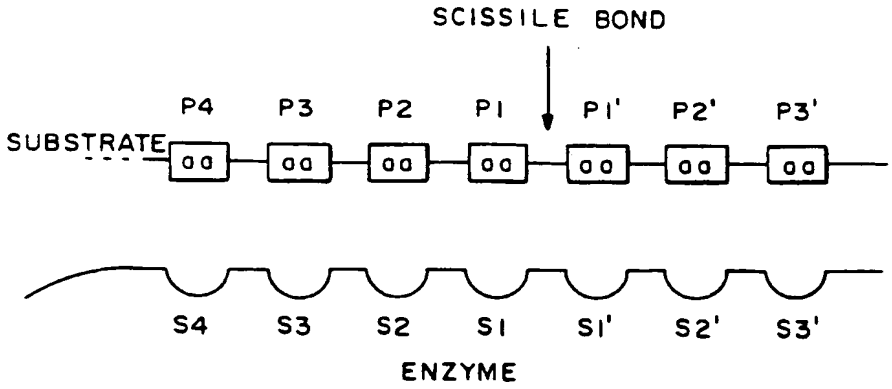

FIG.—2

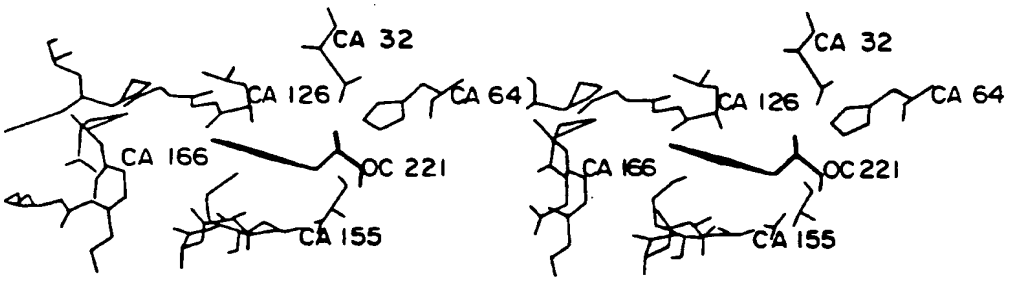

FIG.—3

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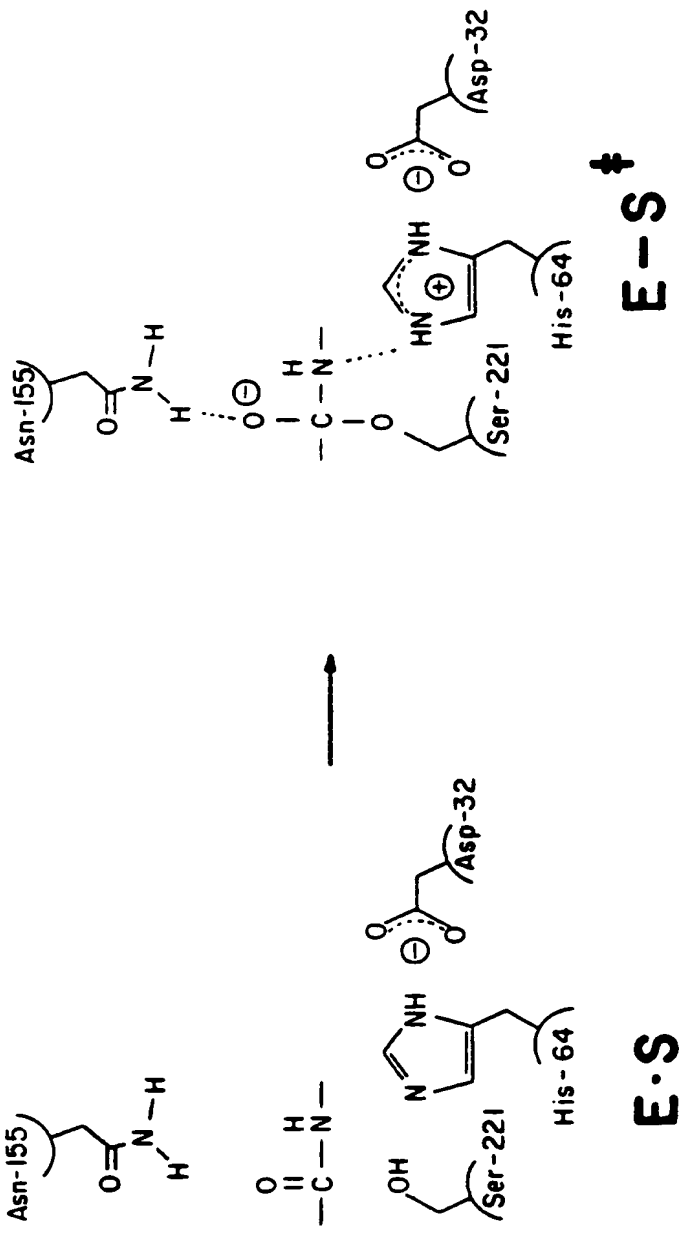

FIG.—4

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Homology of Bacillus proteases

1.Bacillus amyloliquifaciens
2.Bacillus subtilis var.I168
3.Bacillus licheniformis (carlsbergensis)

1									10									20
A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q	G

21									30									40
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H	P

41									50									60
D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D
D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D
D	L	N	V	V	G	G	A	S	F	V	A	G	E	A	Y	N	T	•	D

61									70									80
N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G
G	S	S	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G
G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	D	N	T	T	G

81									90									100
V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G
V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G
V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G

101									110									120
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M	D
S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	T	N	G	M	D

FIG.—5A-1

121									130									140
V	I	N	M	S	L	G	G	P	S	G	S	A	A	L	K	A	A	V	D
V	I	N	M	S	L	G	G	P	T	G	S	T	A	L	K	T	V	V	D
V	I	N	M	S	L	G	G	A	S	G	S	T	A	M	K	Q	A	V	D

141									150									160
K	A	V	A	S	G	V	V	V	V	A	A	A	G	N	E	G	T	S	G
K	A	V	S	S	G	I	V	V	A	A	A	A	G	N	E	G	S	S	G
N	A	Y	A	R	G	V	V	V	V	A	A	A	G	N	S	G	N	S	G

161									170									180
S	S	S	T	V	G	Y	P	G	K	Y	P	S	V	I	A	V	G	A	V
S	T	S	T	V	G	Y	P	A	K	Y	P	S	T	I	A	V	G	A	V
S	T	N	T	I	G	Y	P	A	K	Y	D	S	V	I	A	V	G	A	V

181									190									200
D	S	S	N	Q	R	A	S	F	S	S	V	G	P	E	L	D	V	M	A
N	S	S	N	Q	R	A	S	F	S	S	A	G	S	E	L	D	V	M	A
D	S	N	S	N	R	A	S	F	S	S	V	G	A	E	L	E	V	M	A

201									210									220
P	G	V	S	I	Q	S	T	L	P	G	N	K	Y	G	A	Y	N	G	T
P	G	V	S	I	Q	S	T	L	P	G	G	T	Y	G	A	Y	N	G	T
P	G	A	G	V	Y	S	T	Y	P	T	N	T	Y	A	T	L	N	G	T

221									230									240
S	M	A	S	P	M	V	A	G	A	A	A	L	I	L	S	K	M	P	N
S	M	A	T	P	M	V	A	G	A	A	A	L	I	L	S	K	M	P	T
S	M	A	S	P	M	V	A	G	A	A	A	L	I	L	S	K	M	P	N

241									250									260
V	T	N	T	Q	V	R	S	S	L	E	N	T	T	T	K	L	G	D	S
V	T	N	A	Q	V	R	D	R	L	E	S	T	A	T	Y	L	G	N	S
L	S	A	S	Q	V	R	N	R	L	S	S	T	A	T	Y	L	G	S	S

261									270					
F	Y	Y	G	K	G	L	I	N	V	Q	A	A	A	Q
F	Y	Y	G	K	G	L	I	N	V	Q	A	A	A	Q
F	Y	Y	G	K	G	L	I	N	V	E	A	A	A	Q

FIG.— 5A—2

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ALIGNMENT OF B.AMYLOLIQUIFACIENS SUBTILISIN AND THERMITASE
1.B.amyloliquifaciens subtilisin
2.thermitase

1																10			
A	Q	S	V	*	P	Y	*	*	*	*	*	*	G	V	S	Q	I	K	A
Y	T	P	N	D	P	Y	F	S	S	R	Q	Y	G	P	Q	K	I	Q	A
						20										30			
P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S
P	Q	A	W	D	I	A	E	*	G	S	G	A	K	I	A	I	V	D	T
						40												50	
G	I	D	S	S	H	P	D	L	*	*	K	V	A	G	G	A	S	M	V
G	V	Q	S	N	H	P	D	L	A	G	K	V	V	G	G	W	D	F	V
								60										70	
P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	G	T
D	N	D	S	T	P	*	Q	N	G	N	G	H	G	T	H	C	A	G	I
									80										90
V	A	A	L	*	N	N	S	I	G	V	L	G	V	A	P	S	A	S	L
A	A	A	V	T	N	N	S	T	G	I	A	G	T	A	P	K	A	S	I
									100										110
Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G
L	A	V	R	V	L	D	N	S	G	S	G	T	W	T	A	V	A	N	G
									120										130
I	E	W	A	I	A	N	N	M	D	V	I	N	M	S	L	G	G	P	S
I	T	Y	A	A	D	Q	G	A	K	V	I	S	L	S	L	G	G	T	V
									140										150
G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	V
G	N	S	G	L	Q	Q	A	V	N	Y	A	W	N	K	G	S	V	V	V

FIG.—5B—1

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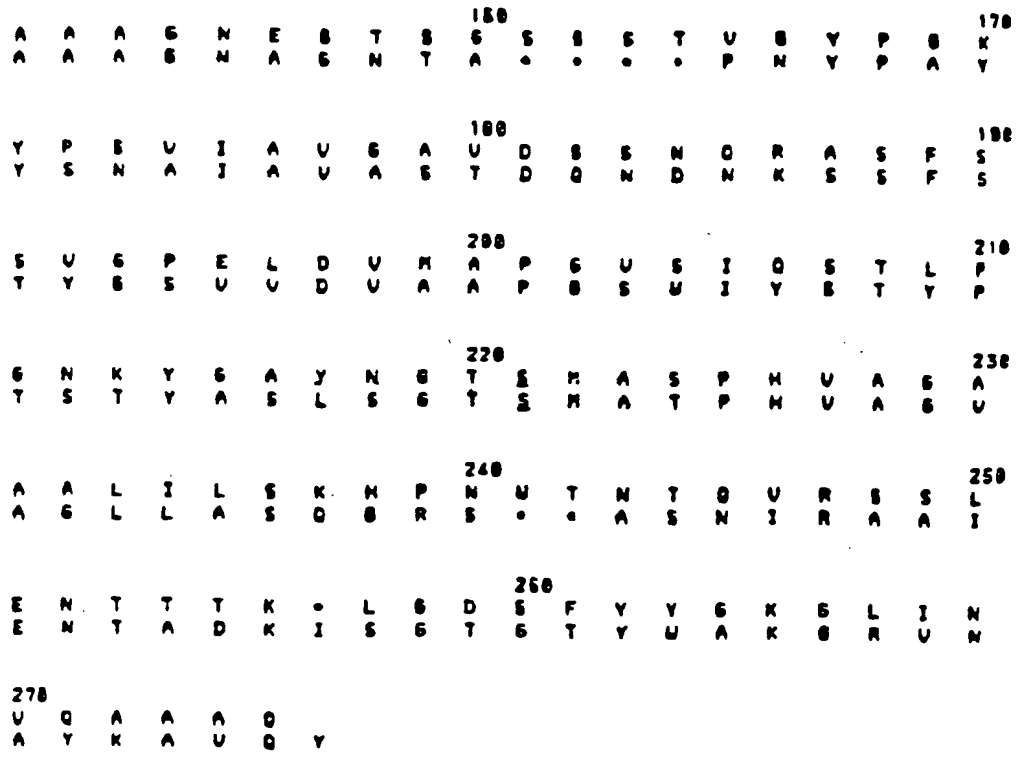

FIG.—5B-2

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TOTALLY CONSERVED RESIDUES IN SUBTILISINS

1									10											20
.	.	.	.	P	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
21									30											40
.	.	B	.	.	.	.	.	.	.	.	D	.	B	.	.	.	.	H	.	
41									50											60
.	.	.	.	.	B	.	.	.	.	V	.	.	.	.	.	.	.	.	.	
61									70											80
.	.	.	H	G	T	H	.	.	G	.	.	.	.	.	.	.	.	.	.	
81									90											100
.	.	G	.	.	.	.	.	.	.	.	.	.	.	V	L	.	.	.	G	
101									110											120
S	B	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	
121									130											140
.	.	.	.	.	L	B	.	.	.	.	.	.	.	.	.	.	.	.	.	
141									150											160
.	.	.	.	.	B	.	.	.	.	.	.	.	G	N	.	.	.	.	.	
161									170											180
.	.	.	.	.	.	Y	P	.	.	.	.	.	.	.	.	U	.	.	.	
181									190											200
.	.	.	.	.	.	.	B	F	S	.	.	.	.	.	.	.	.	.	.	
201									210											220
P	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	T	
221									230											240
S	M	A	.	P	H	U	A	G	.	.	.	.	.	.	.	.	.	.	.	
241									250											260
.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	
261									270											
.	.	.	.	.	.	.	.	N	.	.	.	.	.	.						

FIG.—5C

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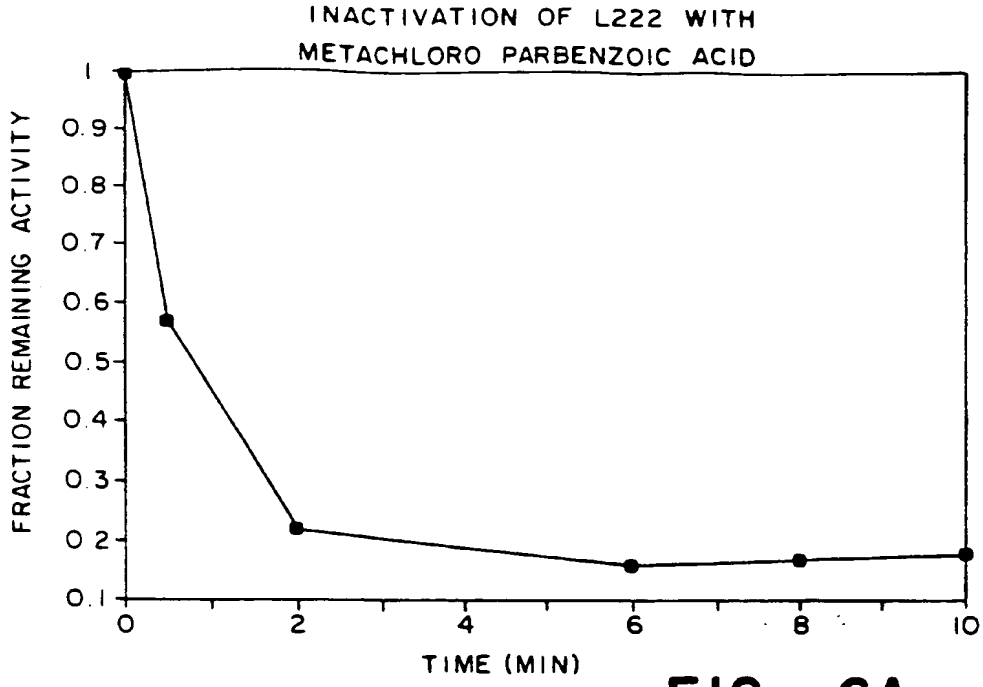

FIG.—6A

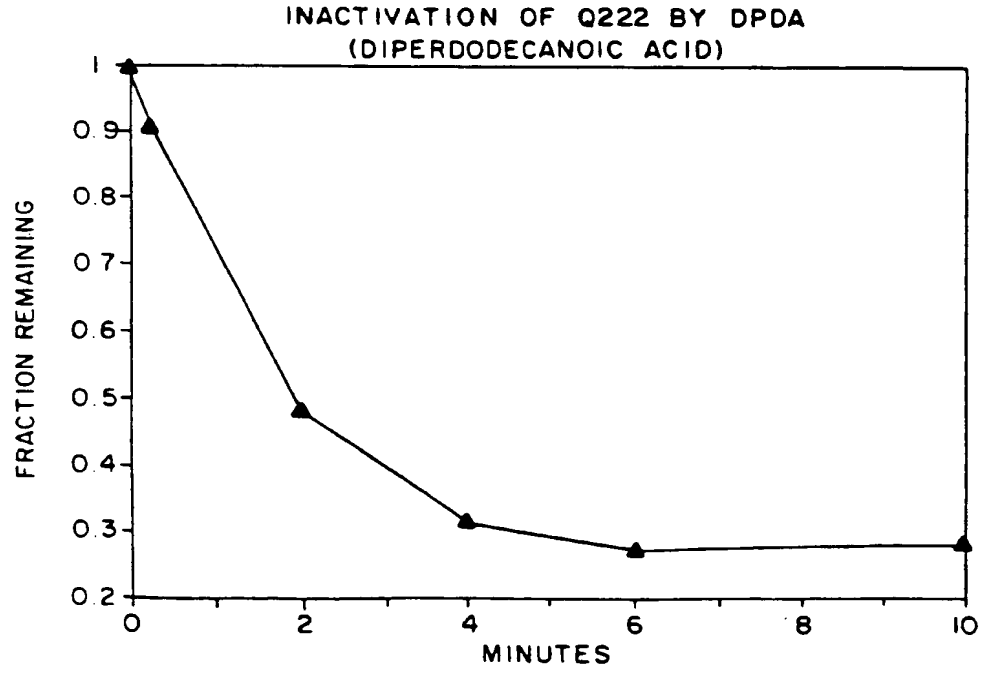

FIG.—6B

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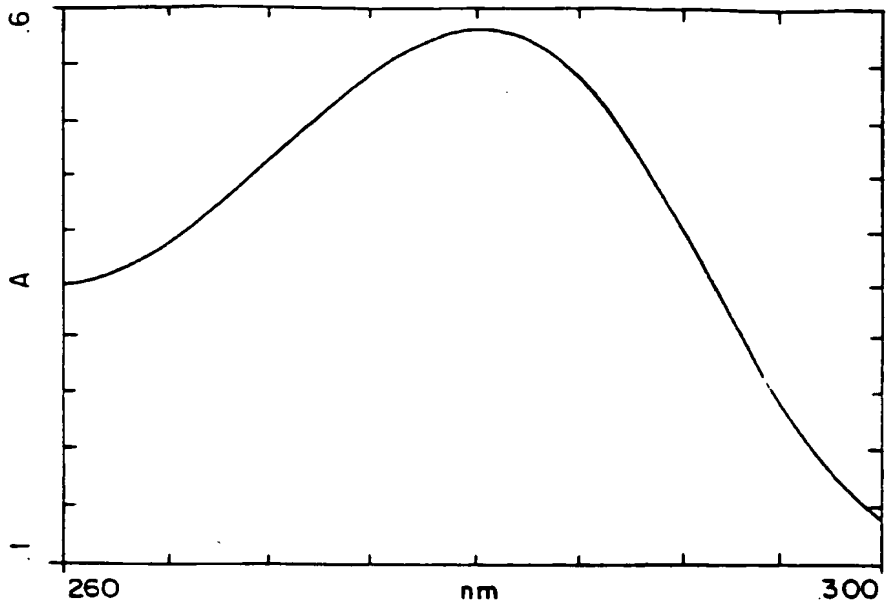

FIG. —7A

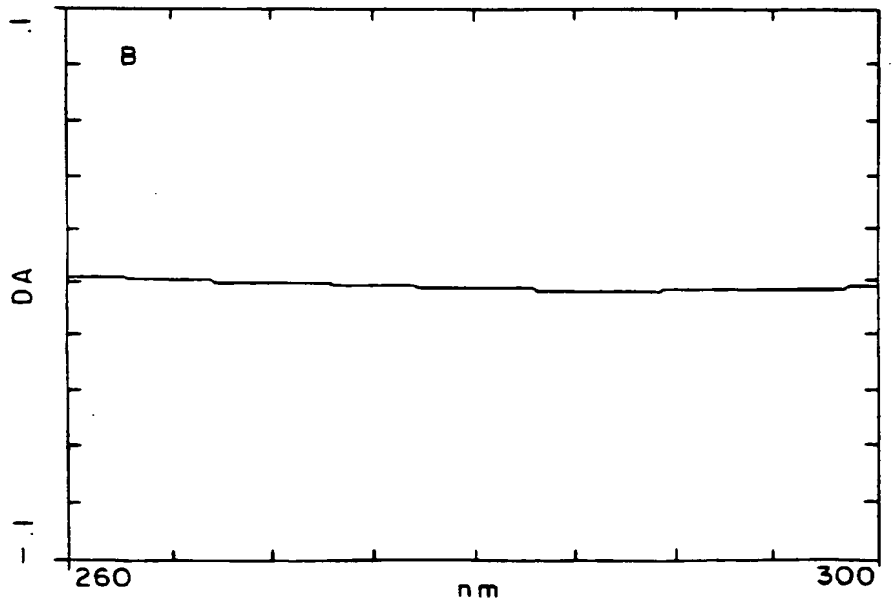

FIG. — 7B

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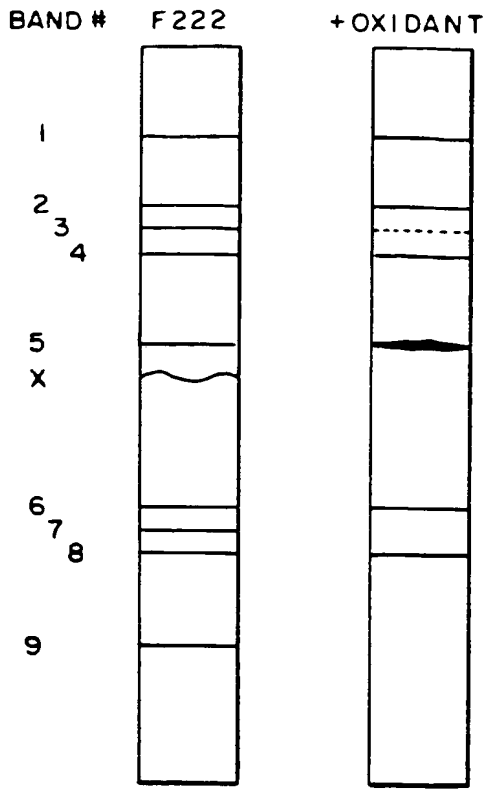

FIG.— 8

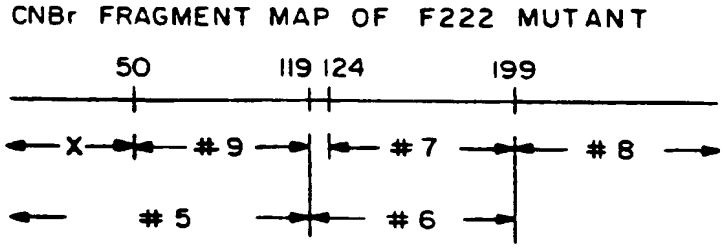

FIG.—9

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1. Codon number: 43 45

2. Wild type amino acid sequence: Lys-Val-Ala-Gly-Gly-Ala-Ser-Met-Val-Pro-Ser

3. Wild type DNA sequence:
```
5'-AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTT-CCT-TCT
   TTC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAA-GGA-AGA-5'
```

4. pΔ50:
```
        ** *                              *
5'-AAG-GCC-T----------------GC-ATG-GTA-CCT-TCT
   TTC-CGG-A----------------CG-TAC-CAT-GGA-AGA-5'
      Stu I                              Kpn I
```

5. pΔ50 cut with *Stu I/Kpn I*
```
                                          *
5'-AAG-G                                pCT-TCT
   TTC-Cp                            CAT-GGA-AGA-5'
```

6. Cut pΔ50 ligated with cassettes:
```
                                         *
5'-AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTA-CCT-TCT
   TCC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAT-GGA-AGA-5'
```

7. Mutagenesis primer for pΔ50:
```
                          ***          *
5'-CT-GAT-TTA-AAG-GCC-TGC-ATG-GTA-CCT-TCT-GA
```

8. Mutants made: V45, P45, V45/P48, E46, E48, V48, C49, C50, F50

FIG.—10

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1. Codon number: 117 120 124 126 130

2. Wild type amino acid sequence: Asn-Asn-Met-Asp-Val-Ile-Asn-Met-Ser-Leu-Gly-Gly-Pro-Ser

3. Wild type DNA sequence:
5'-AAC-AAT-ATG-GAC-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGA-CCT-TCT
TTG-TTA-TAC-CTG-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCT-GGA-AGA-5'

4. pΔ124:
5'-AAC-AAT-ATG-GAT-ATC----------C-GGG-GGC-CCT-TCT
TTG-TTA-TAC-CTA-TAG----------G-CCC-CCG-GGA-AGA-5'
Eco RV *Apa* I

5. pΔ124 cut with *Eco* RV and *Apa* I
5'-AAC-AAT-ATG-GAT pCT-TCT
TTG-TTA-TAC-CTAp CCG-GGA-AGA-5'

6. Cut pΔ124 ligated with cassettes:
5'-AAC-AAT-ATG-GAT-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGC-CCT-TCT
TTG-TTA-TAC-CTA-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCG-GGA-AGA-5'

7. Mutagenesis primer for pΔ124::
5'-AAC-AAT-ATG-GAT-ATC-C-GGG-GGC-CCT-TCT-GGT-TC-3'

8. Mutants made: I 124, L 124 AND C126

FIG.—11

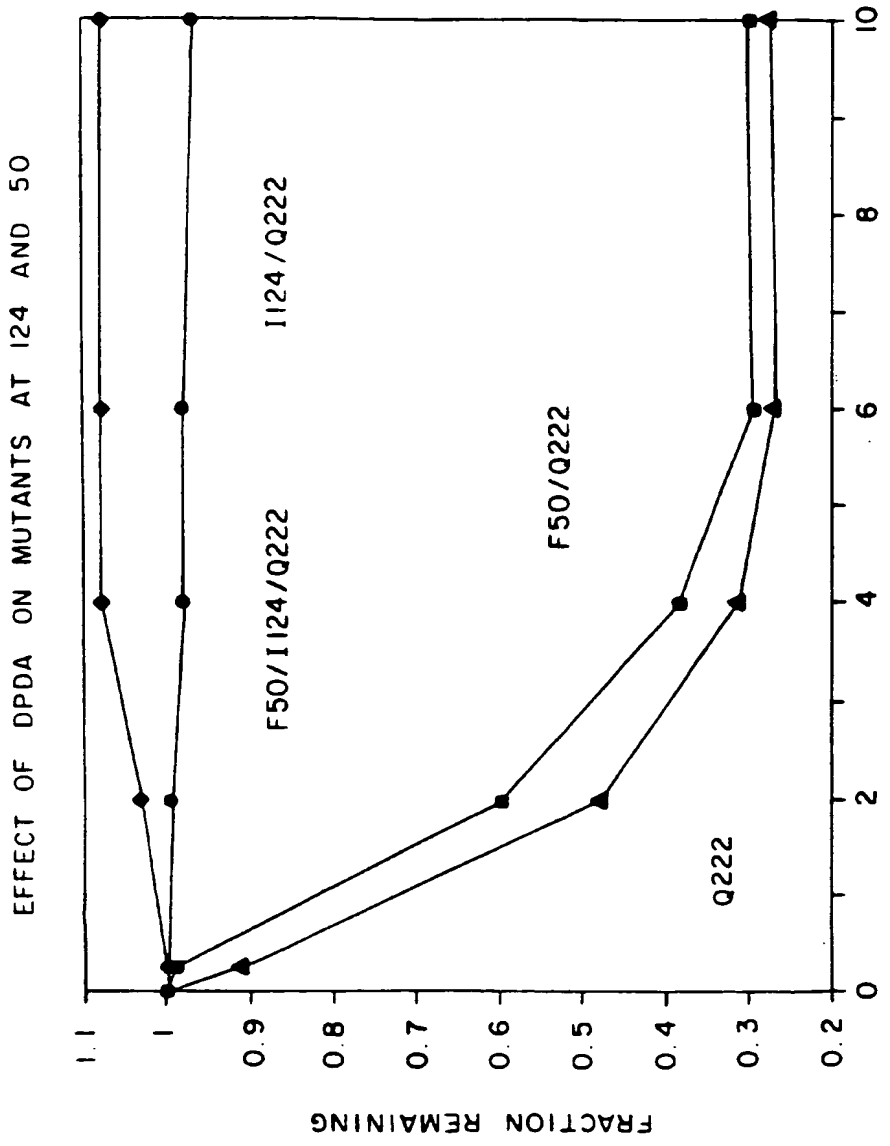

FIG.-12

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Codon:	166
Wild type amino acid sequence:	Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly
1. Wild type DNA sequence:	5'-ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT-3' 3'-TGA AGG CCG TCG AGT TCG TGT CAC CCG ATG GGA CCA-5'
2. pA166 DNA sequence:	5'-ACT TCC GGG AGC TCA A - - - - - - - - - C CCG GGT-3' 3'-TGA AGG CCC TCG AGT T - - - - - - - - - G GGC CCA-5' SacI XmaI
3. pA166 cut with SacI and XmaI:	5'-ACT TCC GGG AGC T pCCG GGT-3' 3'-TGA AGG CCCp CA-5'
4. Cut pA166 ligated with duplex DNA cassette pools:	5'-ACT TCC GGG AGC TCA AGC ACA GTG NNN TAC CCG GGT-3' 3'-TGA AGG CCC TCG AGT TCG TGT CAC NNN ATG GGC CCA-5'

MUTAGENESIS PRIMER 37 MER

5' AA GGC ACT TCC GGG AGC TCA ACC CGG GTA AA TAC CCT 3'

FIG.—13

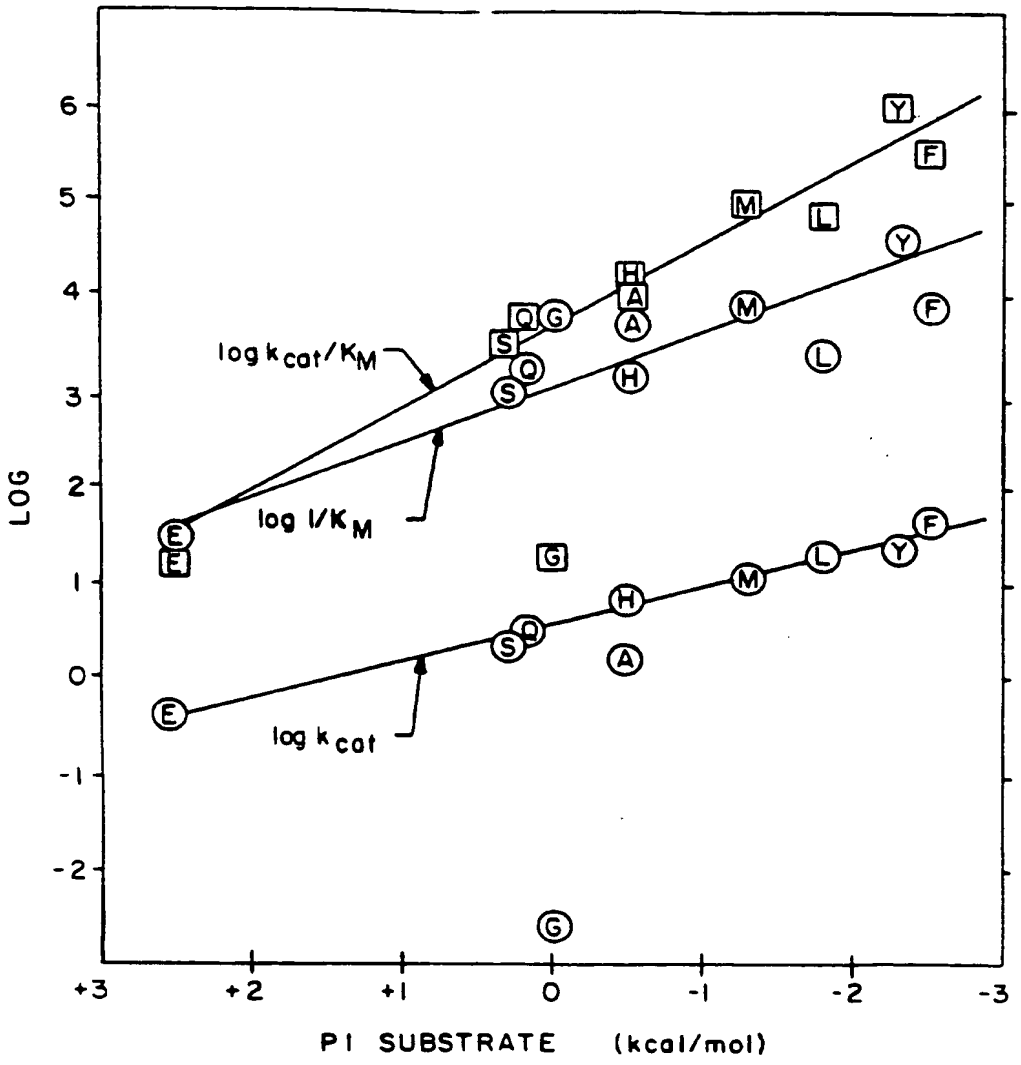

FIG. — 14

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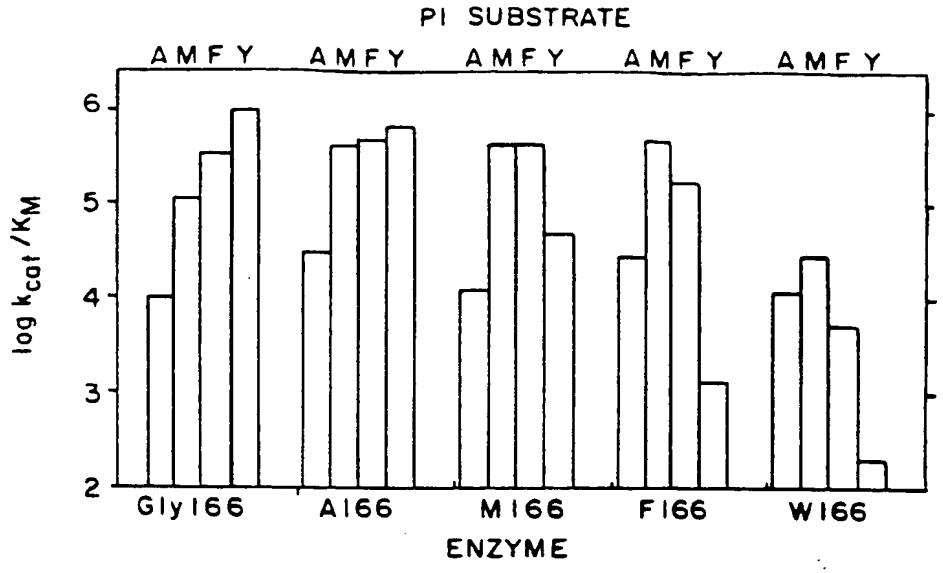

FIG.—15A

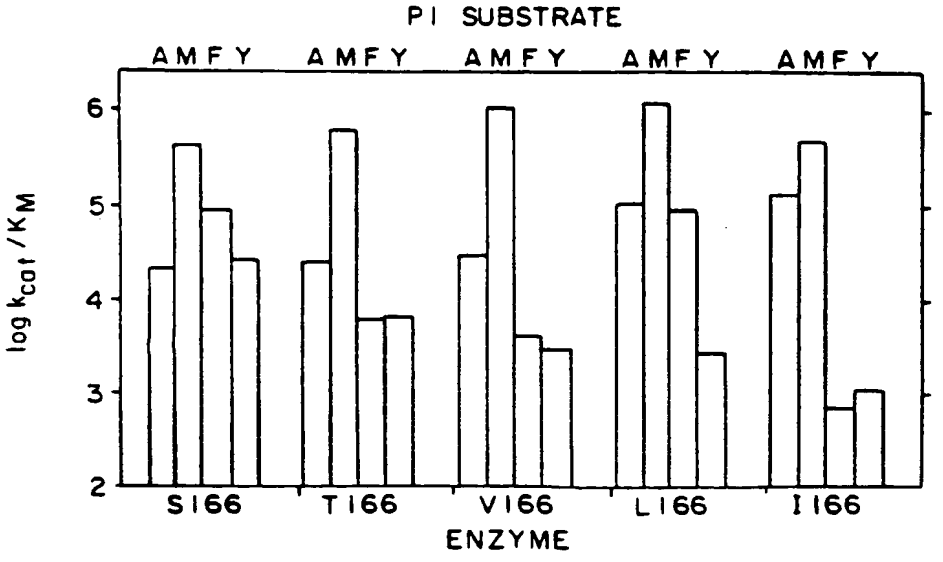

FIG.—15B

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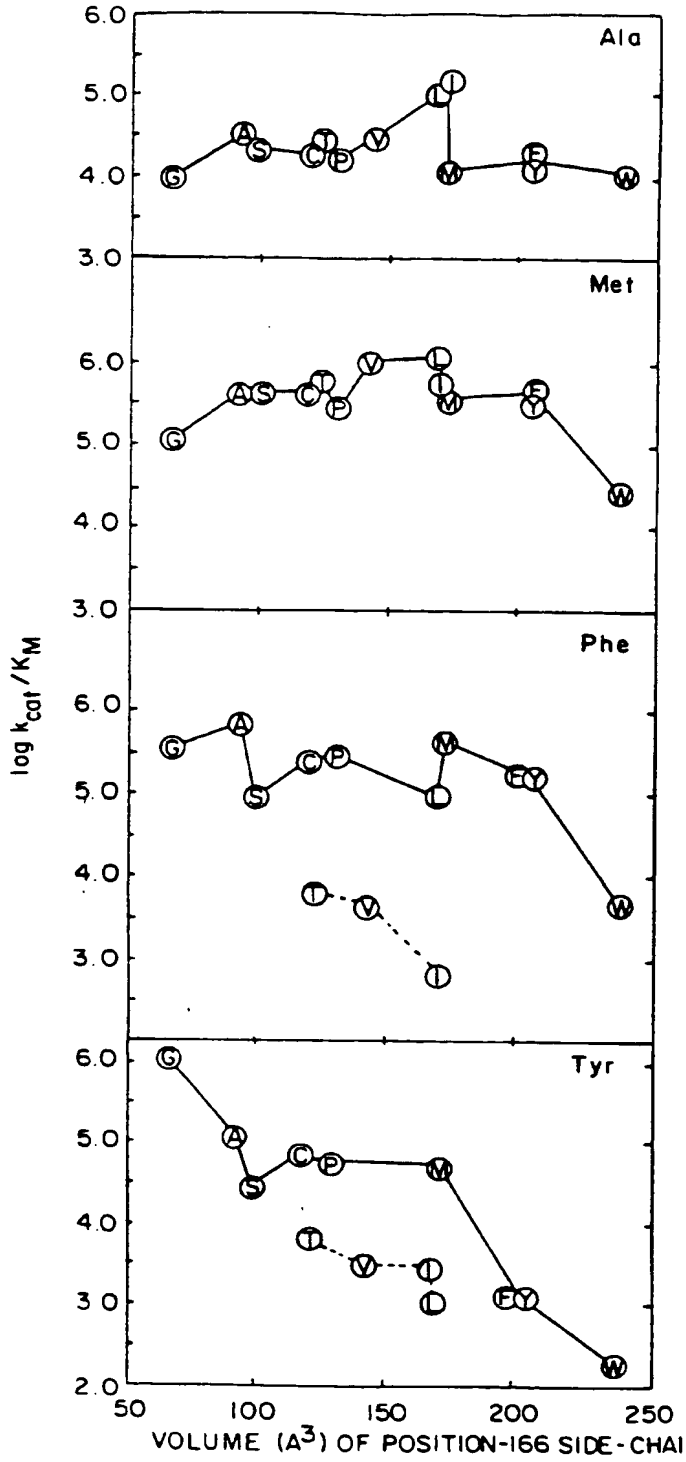

FIG.-16

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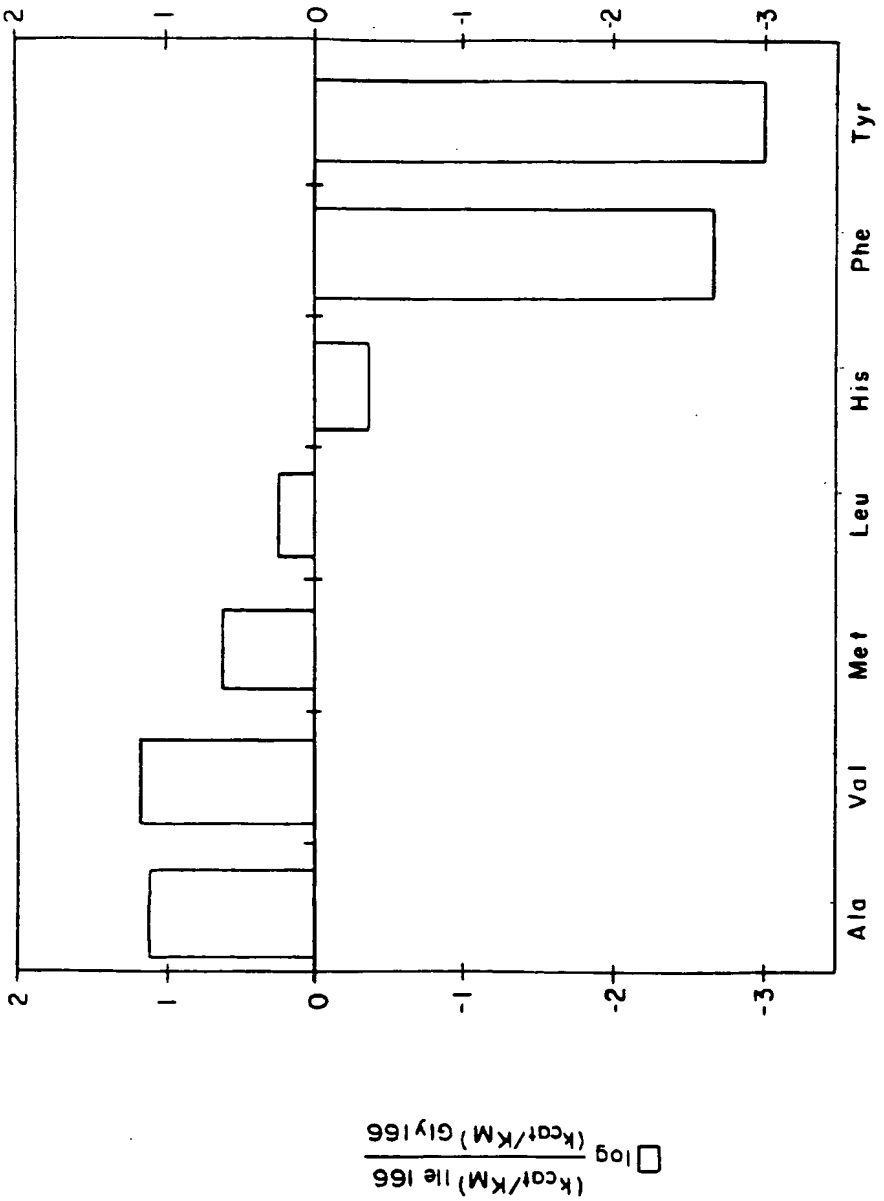

FIG. - 17

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GLY-169 CASSETTE MUTAGENESIS

CODON:		162 169 173
WILD TYPE AMINO ACID SEQUENCE:		SER SER THR VAL GLY TYR PRO GLY LIS TYR PRO SER
1. WILD TYPE DNA SEQUENCE	5'	TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT 3'
	3'	AGT TCG TGT CAC CCG ATG GGA CCA TTT ATG GGA AGA 5'
2. P169 DNA SEQUENCE	5'	TCA AGC ACA GTC GGG TAC CCT-----GA TAT CCT TCT 3'
	3'	AGT TCG TGT CAC CCC ATG GGA CT ATA GGA AGA 5'
		KPNI EcoRV
3. P169 CUT WITH KPNI AND ECORV:	5'	TAC AGC ACA GTC GGG TAC pAT CCT TCT 3'
	3'	AGT TCG TGT CAC CCp TA GGA AGA 5'
4. CUT P169 LIGATED WITH	5'	TAC AGC ACA GTG GGG TAC CCT NNN AAA TAT CCT TGT 3'
OLIGONUCLEOTIDE POOLS	3'	AGT TCG TGT CAC CCC ATG GGA NNN TTT ATA GGA AGA 5'
MUTAGENESIS PRIMER FOR P169	5'	AAG CAC AGT GGG GTA CCC TGA TAT CCT TCT GTC A 3'

FIG.—18

EP 0 251 446 B1

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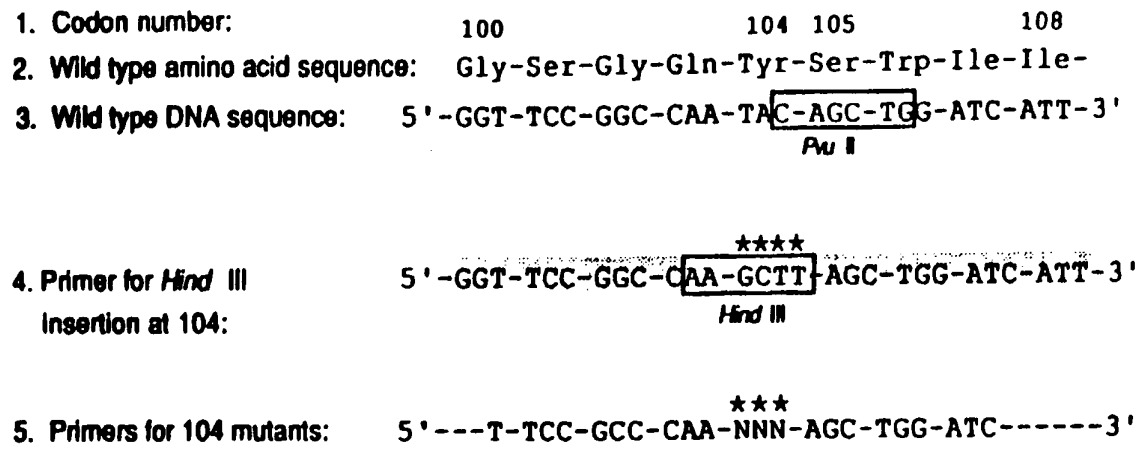

FIG.—19

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1. Codon number: 148 150 152 155
2. Wild type amino acid sequence: Val-Val-Val-Ala-Ala-Ala-Gly-Asn-Glu
3. Wild type DNA sequence: 5'-GTA-GTC-GTT-GCG-GCA-GCC-GGT-AAC-GAA-3'
4. V152/P153 5'-GTA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3' (Kpn I)
5. S 152: 5'-GTA-GTC-GTT-GCG-AGC-GCC-GGT-AAC-GAA-3'
6. G 152: 5'-GTA-GTC-GTT-GCG-GGC-GCC-GGT-AAC-GAA-3'

FIG.—20

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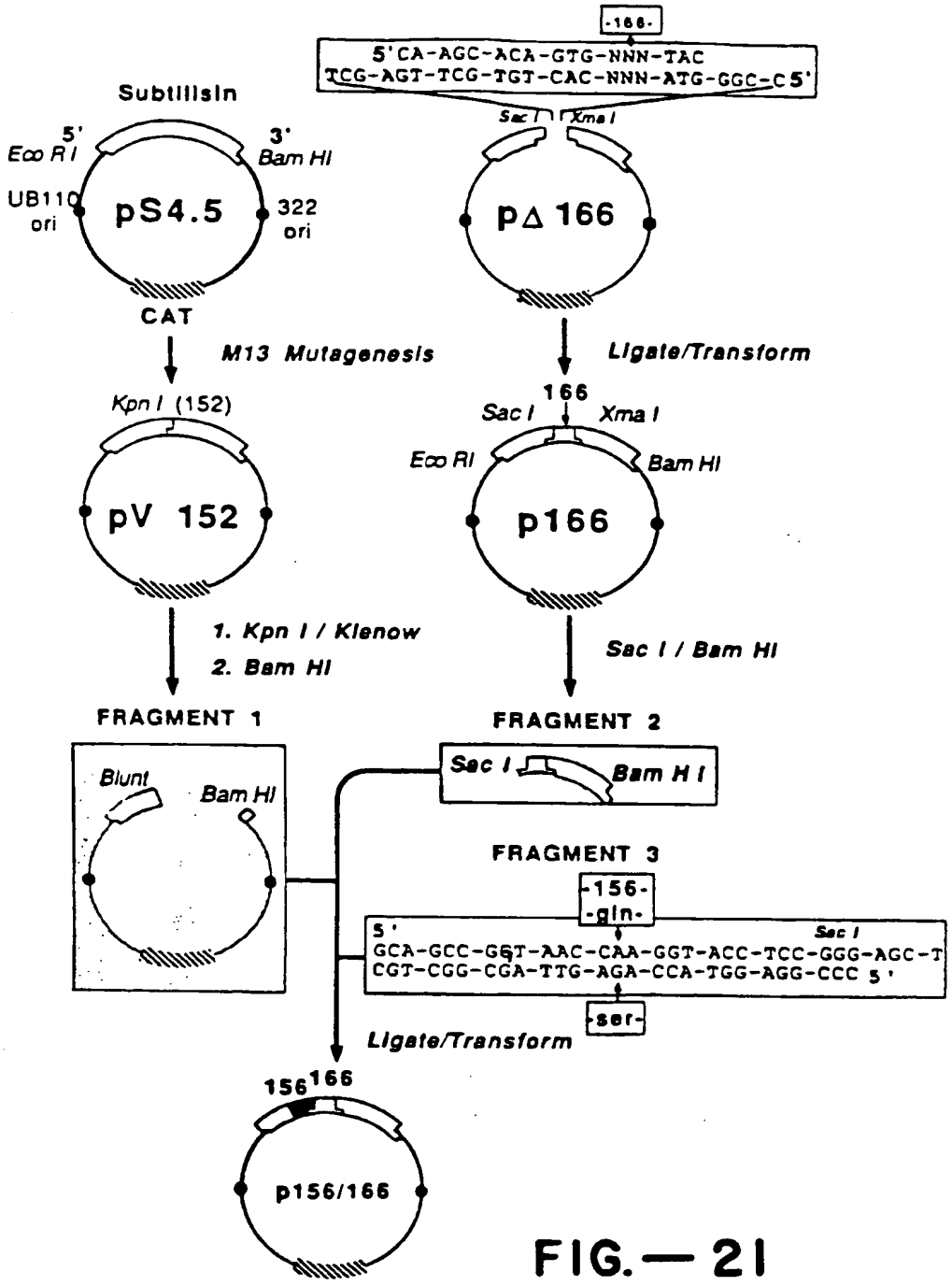

FIG.— 21

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1. Codon number: 211 215 217 220

2. Wild type amino acid sequence: Gly-Asn-Lys-Tyr-Gly-Ala-Tyr-Asn-Gly-Thr-Ser-Met-Ala

3. Wild type DNA sequence:
5'-GGA-AAC-AAA-TAC-GGG-GCG-TAC-AAC-GGT-ACG-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCC-CGC-ATG-TTG-CCA-TGC-AGT-TAC-CGT-5'

4. pΔ217
5'-GGA-AAC-AAA-TAC-GGC-GCC-TAC------GG-ATA-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCG-CGG-ATG------CC-TAT-AGT-TAC-CGT-5'
Nar I *Eco* RV

5. pΔ217 cut with *Nar* I and *Eco* RI
5'-GGA-AAC-AAA-TAC-GG
CCT-TTG-TTT-ATG-CCG-Gp
pA-TCA-ATG-GCA
T-AGT-TAC-CGT-5'

6. Cut pΔ217 ligated with cassettes:
5'-GGA-AAC-AAA-TAC-GGC-GCG-NNN-AAC-GGT-ACA-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCG-CGC-NNN-TTG-CCA-TGT-AGT-TAC-CGT-5'

7. Mutagenesis primer for pΔ217:
5'-GA-AAC-AAA-TAC-GGC-GCC-TAC-GGA-TAT-CAA-TGG-CAT-3'

8. Mutants made: All 19 at 217

FIG.—22

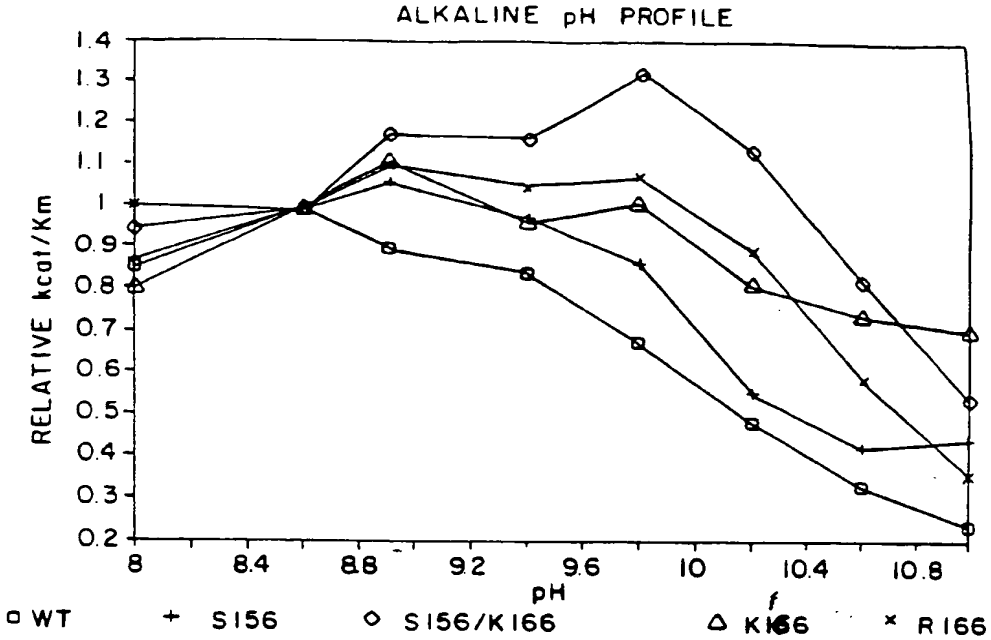

FIG. — 23A

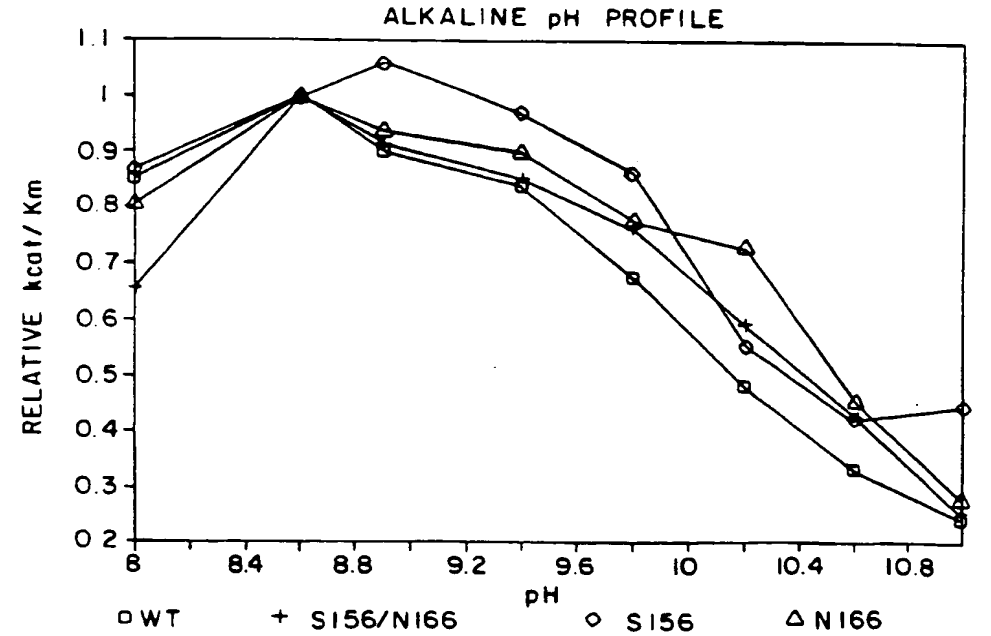

FIG. — 23B

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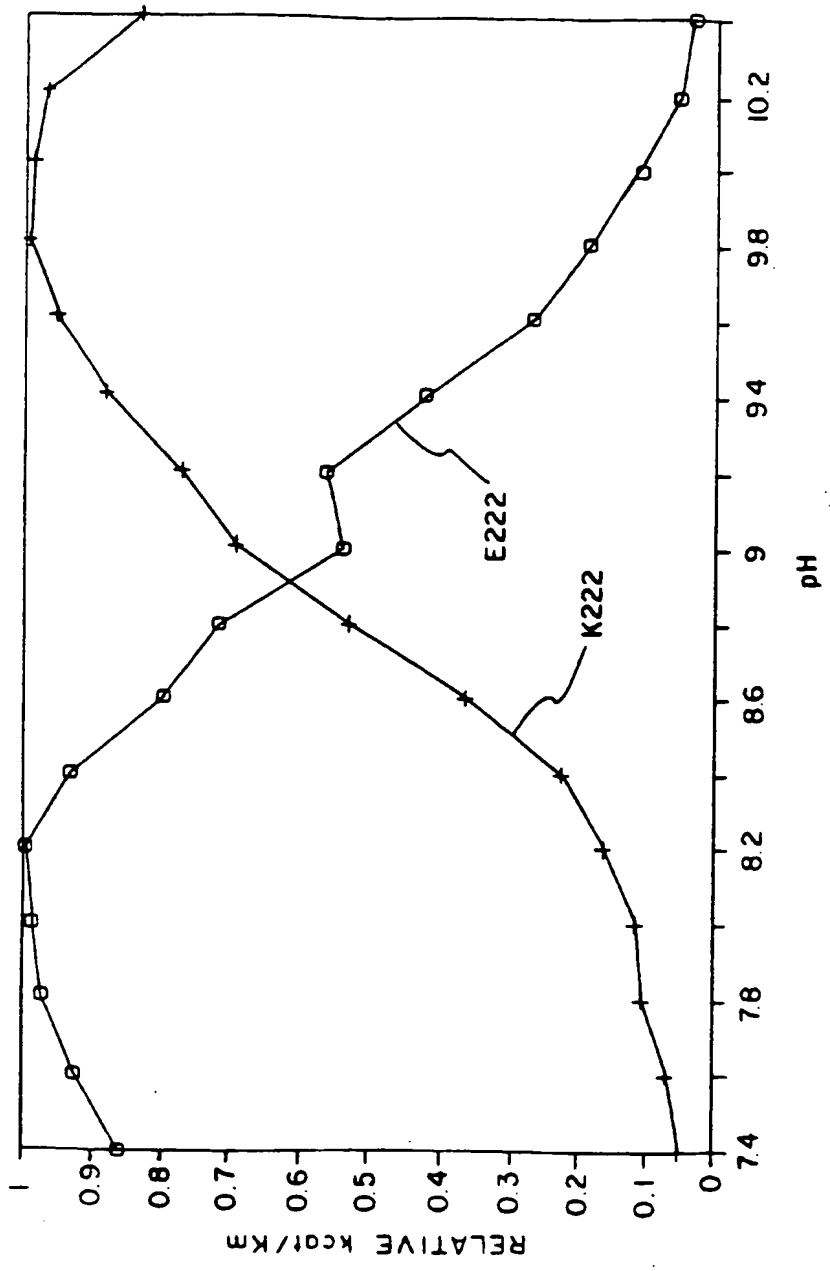

FIG.—24

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1. Codon number: 91 95 100

2. Wild type amino acid sequence: Tyr-Ala-Val-Lys-Val-Leu-Gly-Ala-Asp-Gly-Ser

3. Wild type DNA sequence:
5'-TAC-GCT-GTA-AAA-GTT-CTC-GGT-GCT-GAC-GGT-TCC
ATG-CGA-CAT-TTT-CAA-GAG-CCA-CGA-CTG-CCA-AGG-5'

4. pΔ95:
5'-TAC-GCG-T----------CTC-GCT-GCA-GAC-GGT-TCC
ATG-CGC-A----------GAG-CGA-CGT-CTG-CCA-AGG-5'
Mu I *Pst* I

5. pΔ95 cut with *Mu* I and *Pst* I
5'-TA pGAC-GGT-TCC
ATG-CGCp A-CGT-CTG-CCA-AGG-5'

6. Cut pΔ95 ligated with cassettes:
5'-TAC-GCG-GTA-AAA-GTT-CTC-GGT-GCA-GAC-GGT-TCC
ATG-CGC-CAT-TTT-CAA-GAG-CCA-CGT-CTG-CCA-AGG-5'

7. Mutagenesis primer for pΔ95:
5'-CA-TCA-CTT-TAC-GCG-T-CTC-GCT-GCA-GAC-GGT-TCC

8. Mutants made: C94, C95, D96

FIG.—25

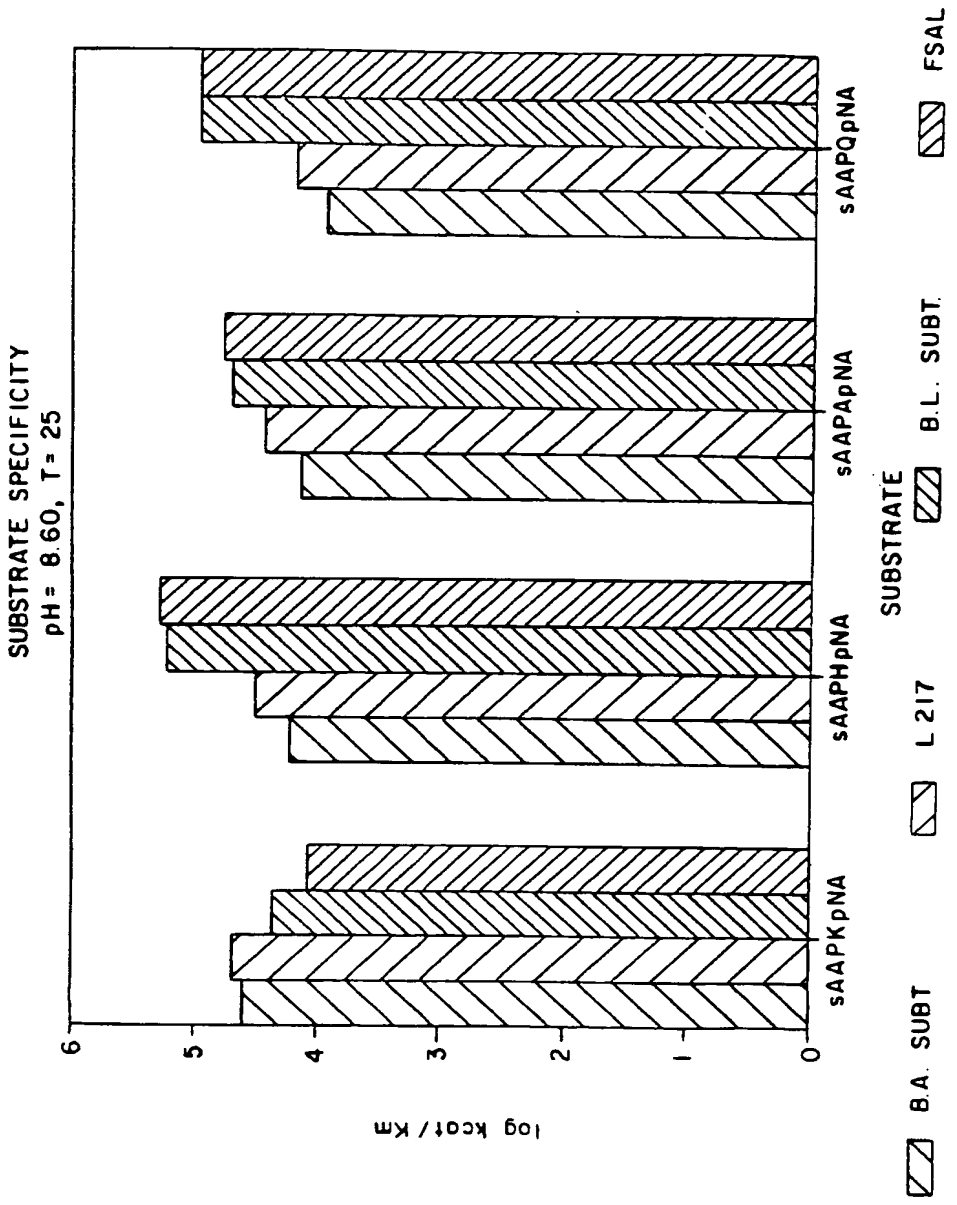

FIG.—26

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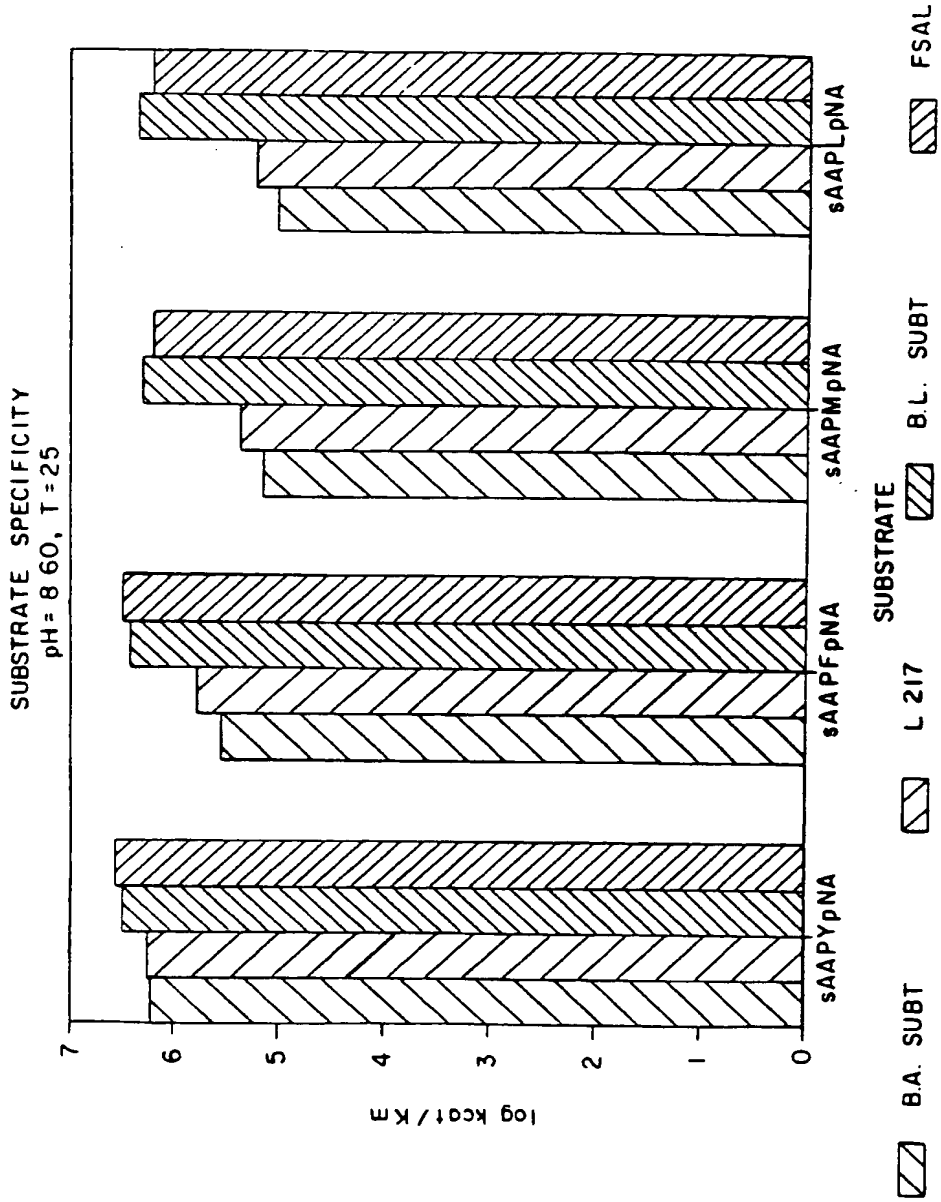

FIG.—27

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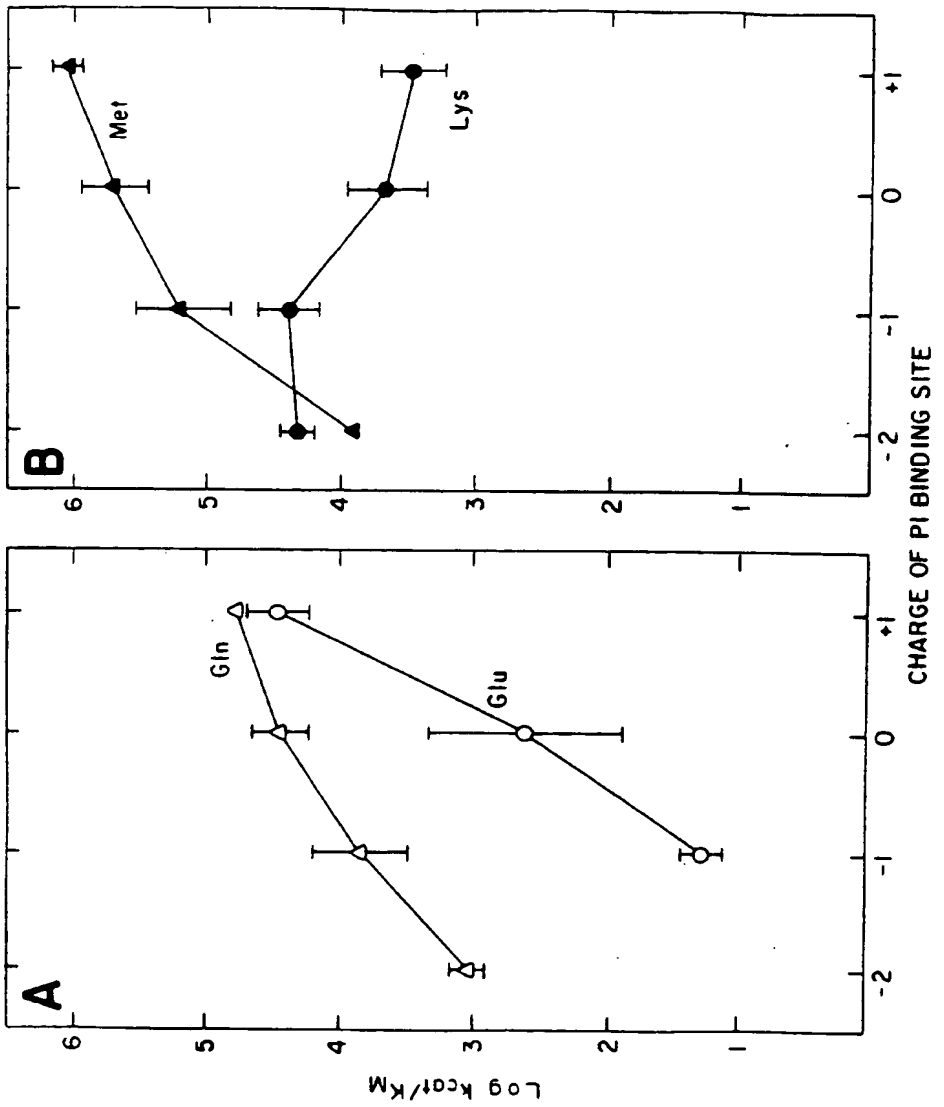

FIG.—28

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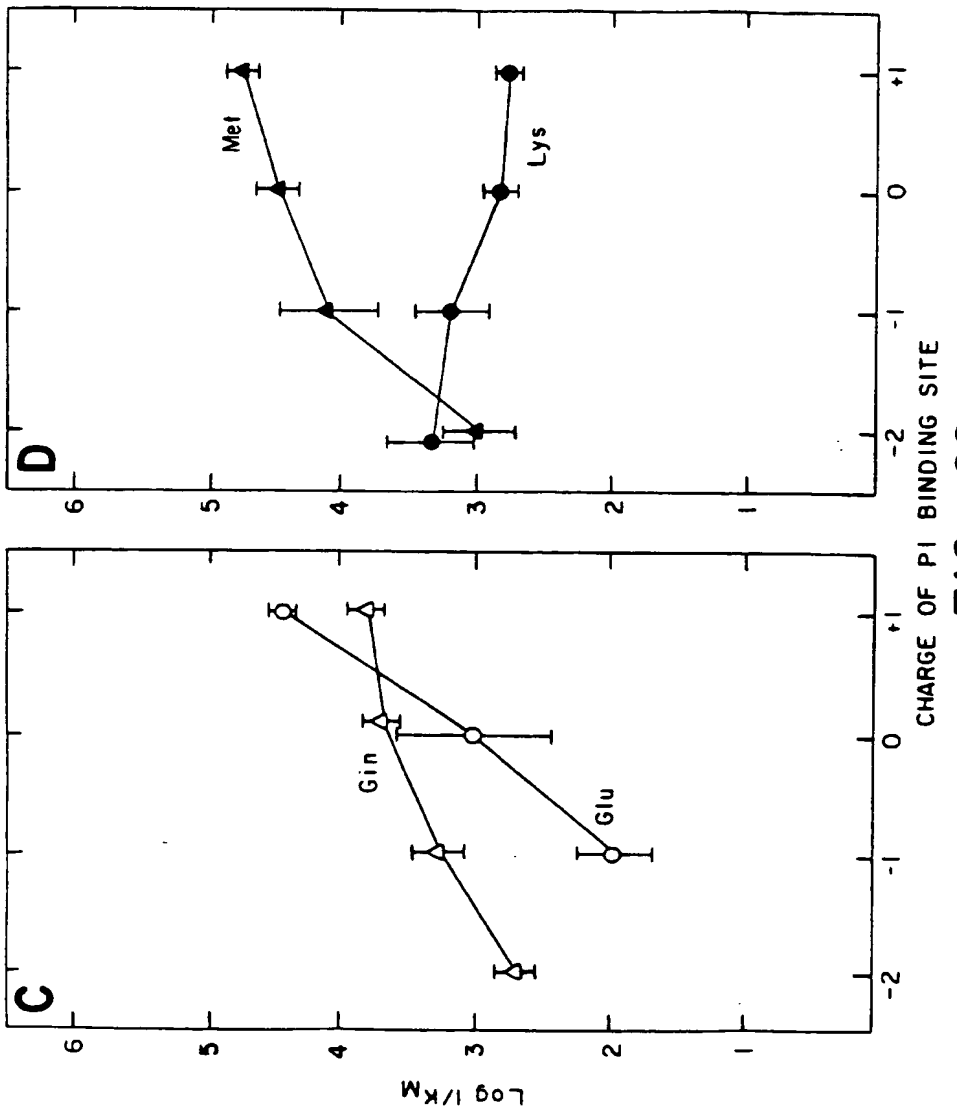

FIG.-28

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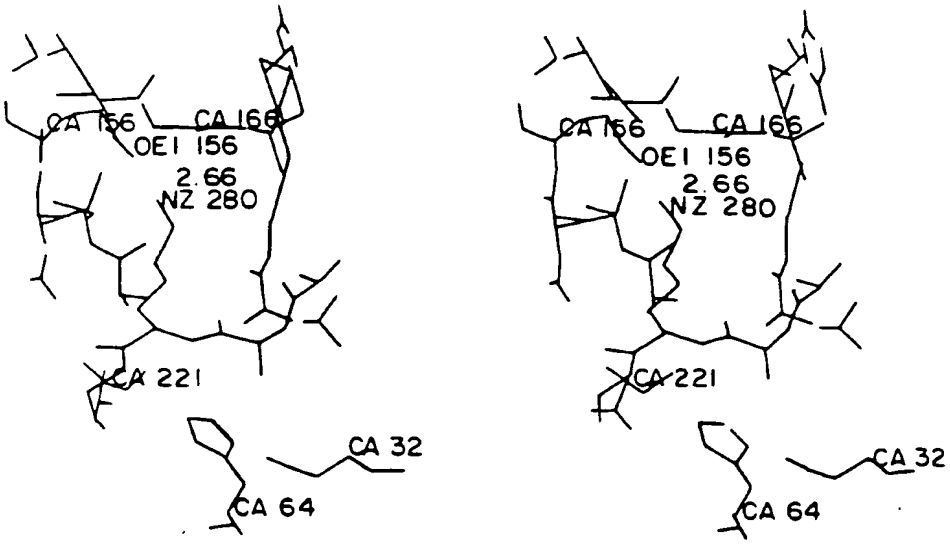

FIG. — 29A

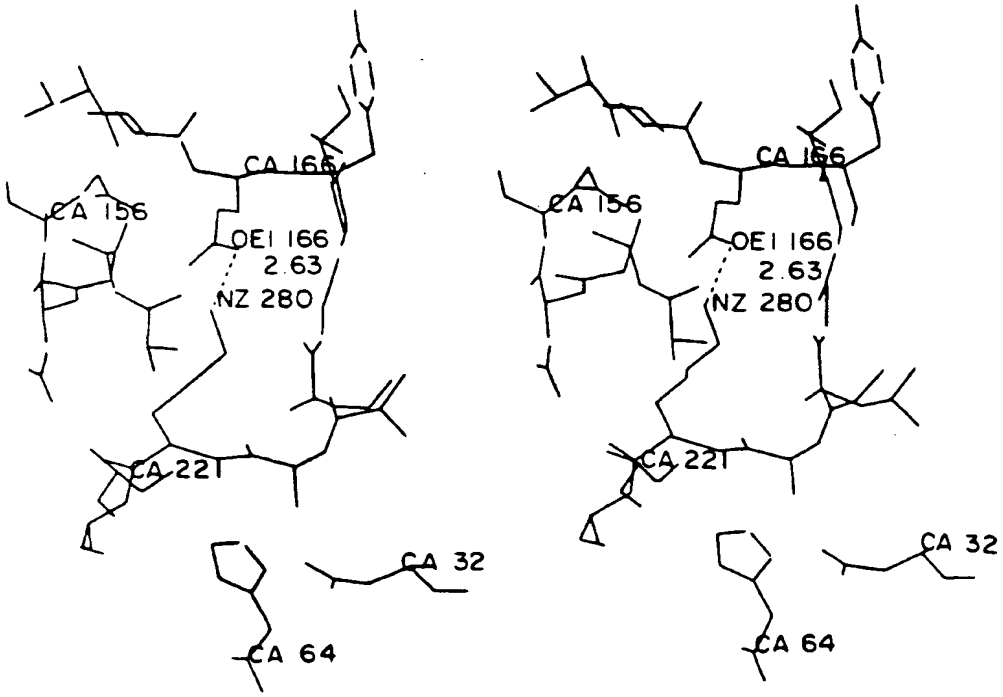

FIG. — 29B

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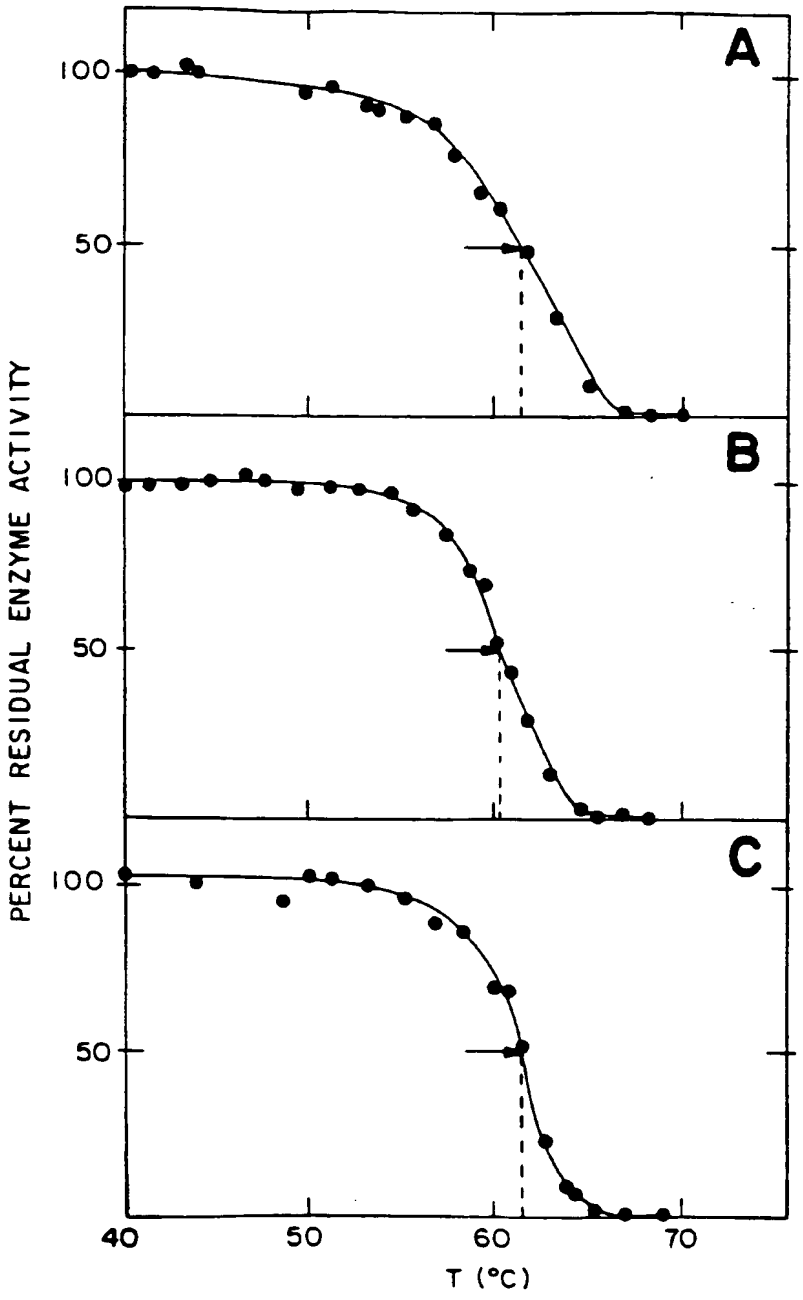

FIG.—30

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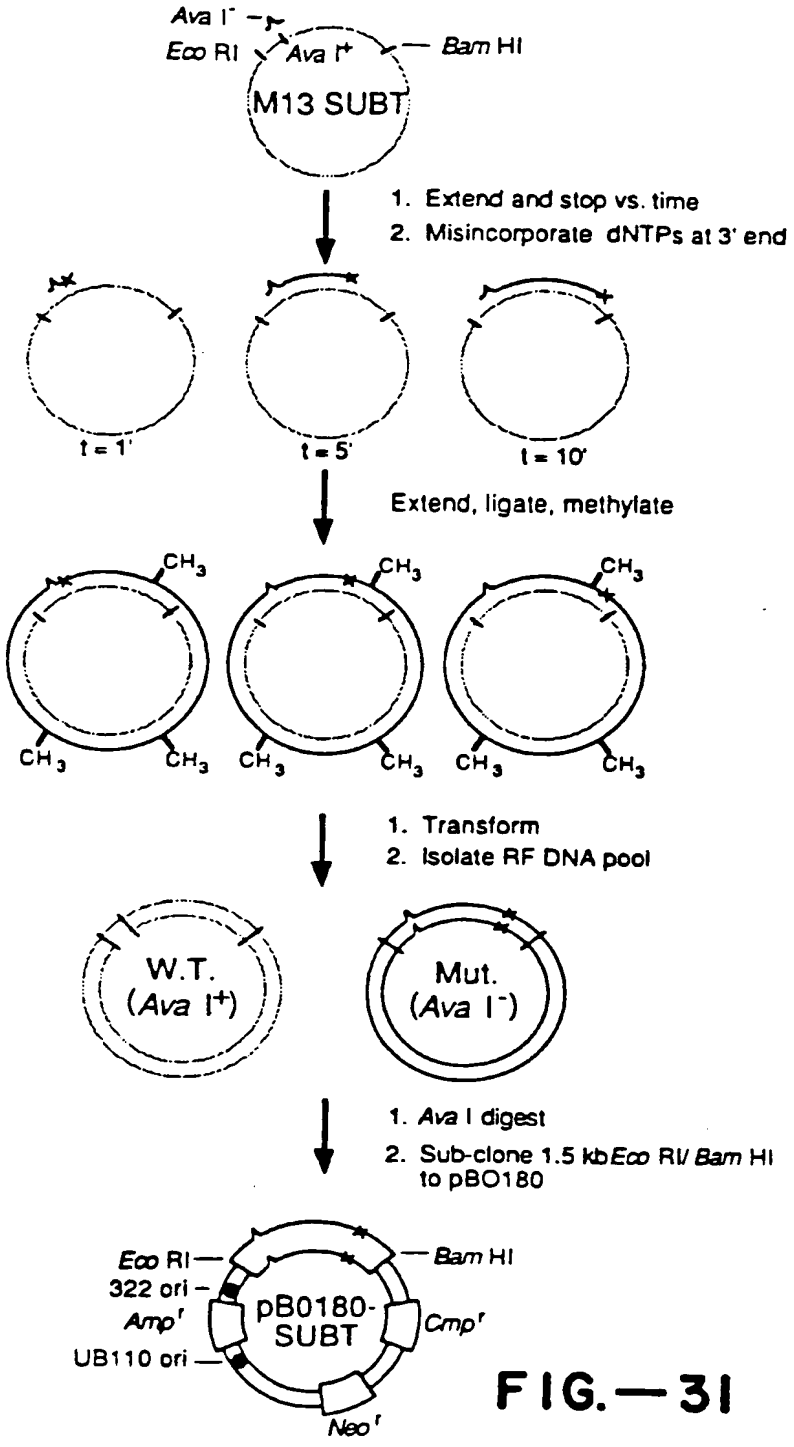

FIG.—31

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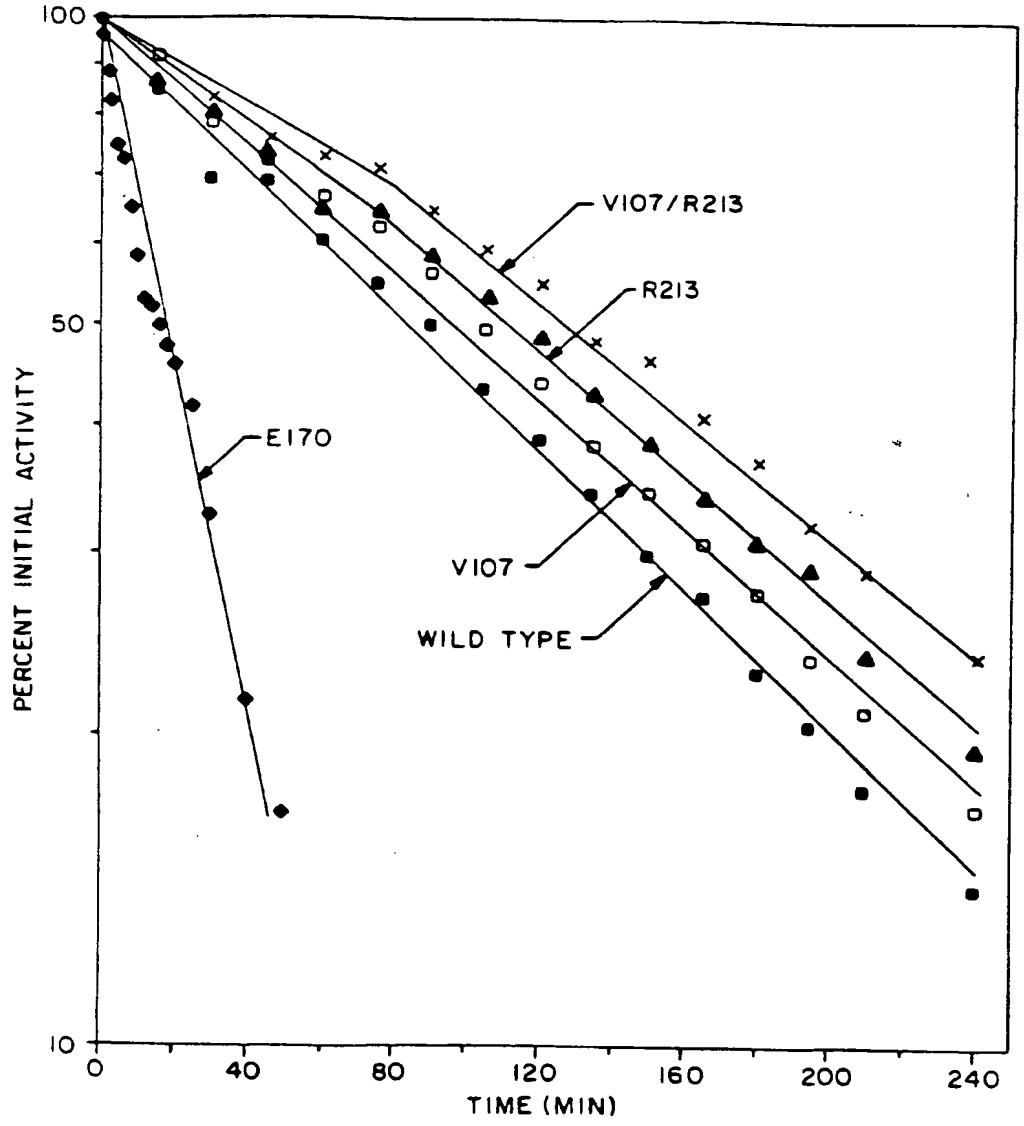

FIG. – 32

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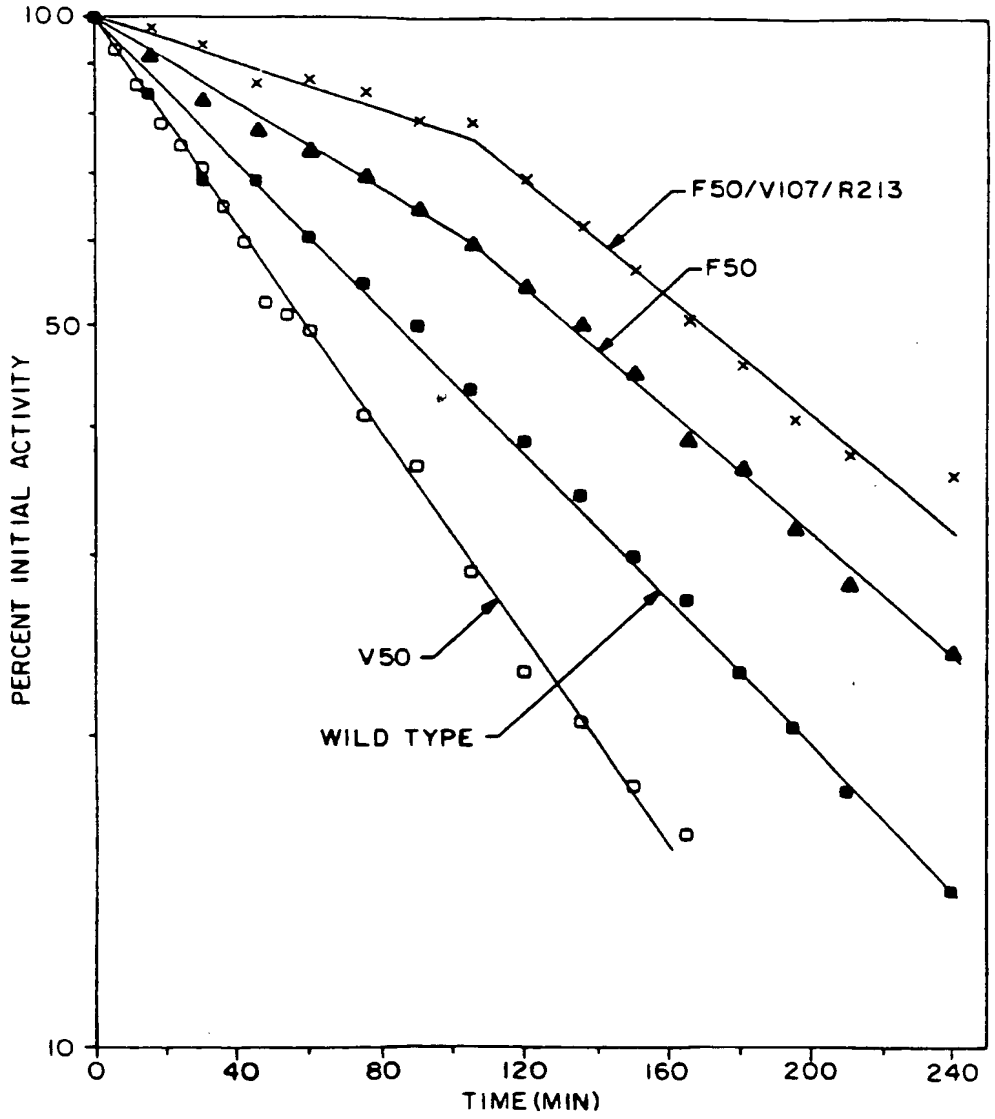

FIG.—33

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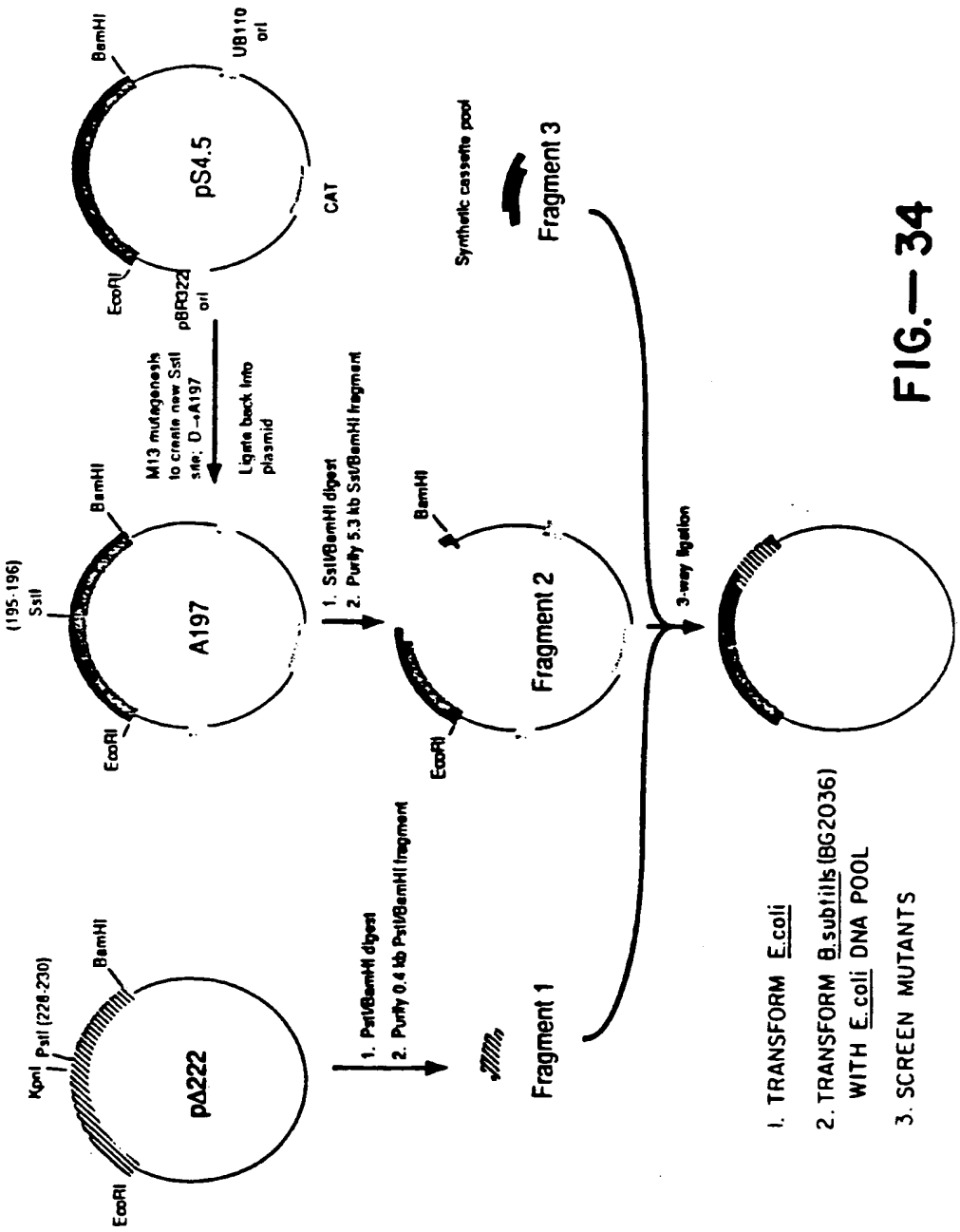

FIG.—34

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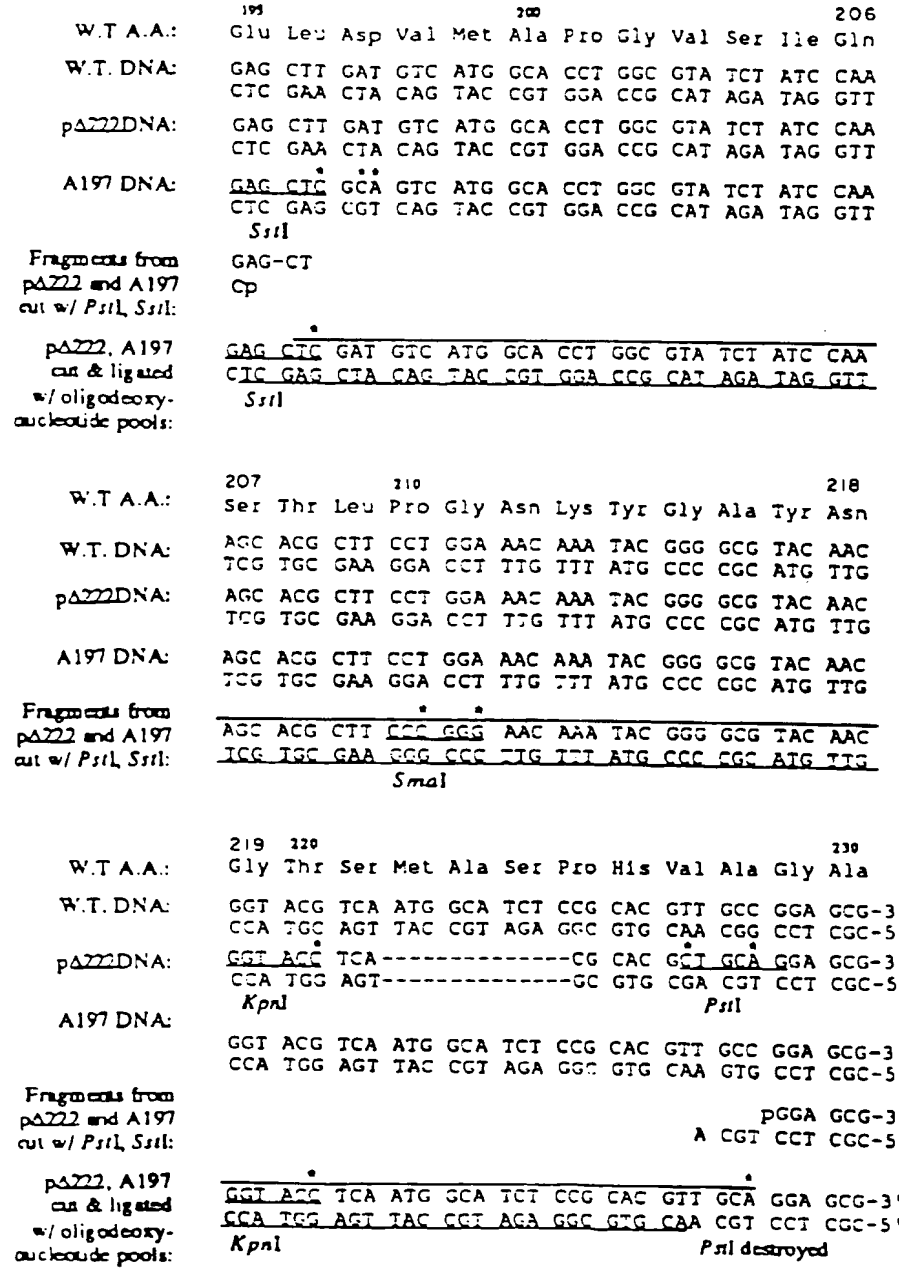

Oligodeoxynucleotide pools synthesized with 2% contaminating nucleotides in each cycle to give ~15% of pool with 0 mutations, ~28% of pool with single mutations, and ~57% of pool with 2 or more mutations, according to the general formula $f = \frac{\mu^n}{n!} e^{-\mu}$.

FIG.—35

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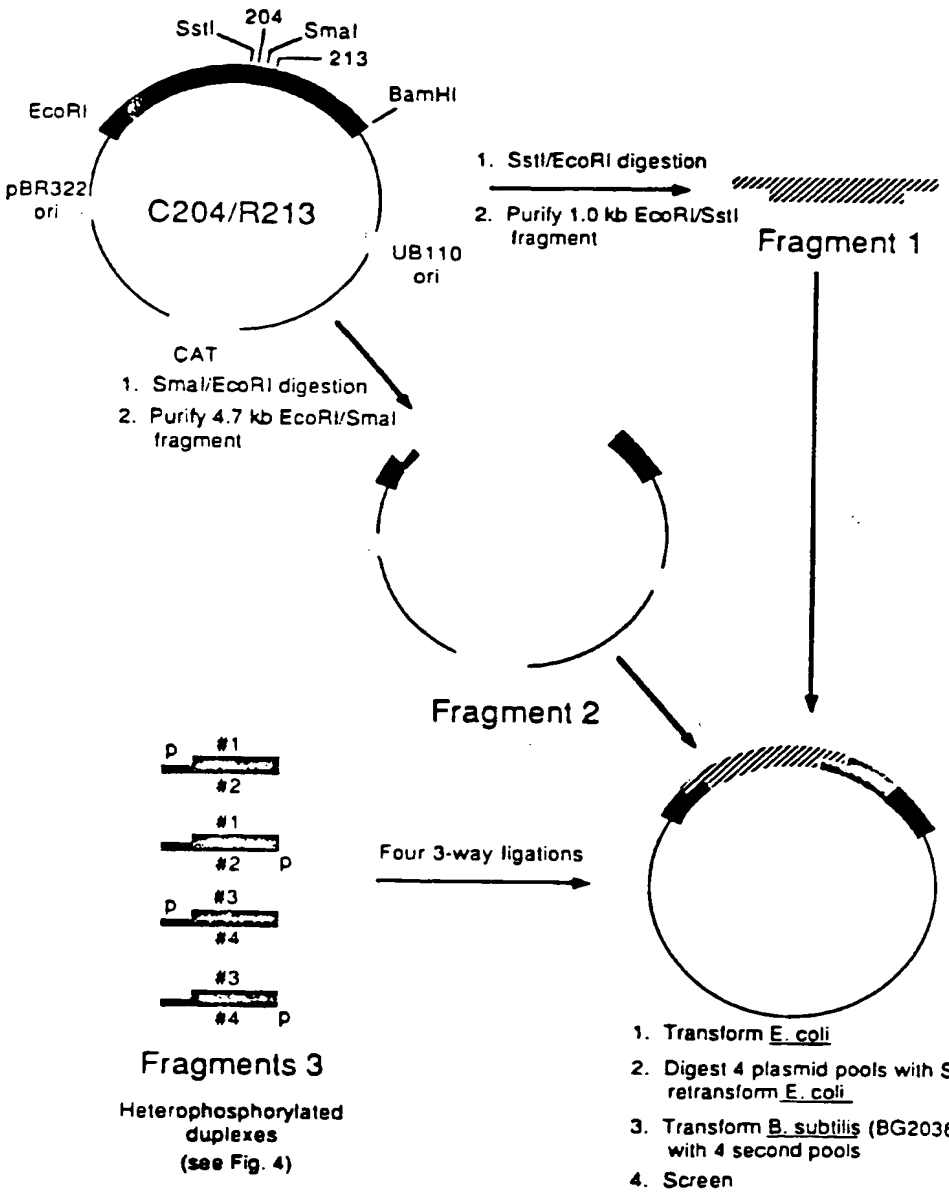

FIG.—36

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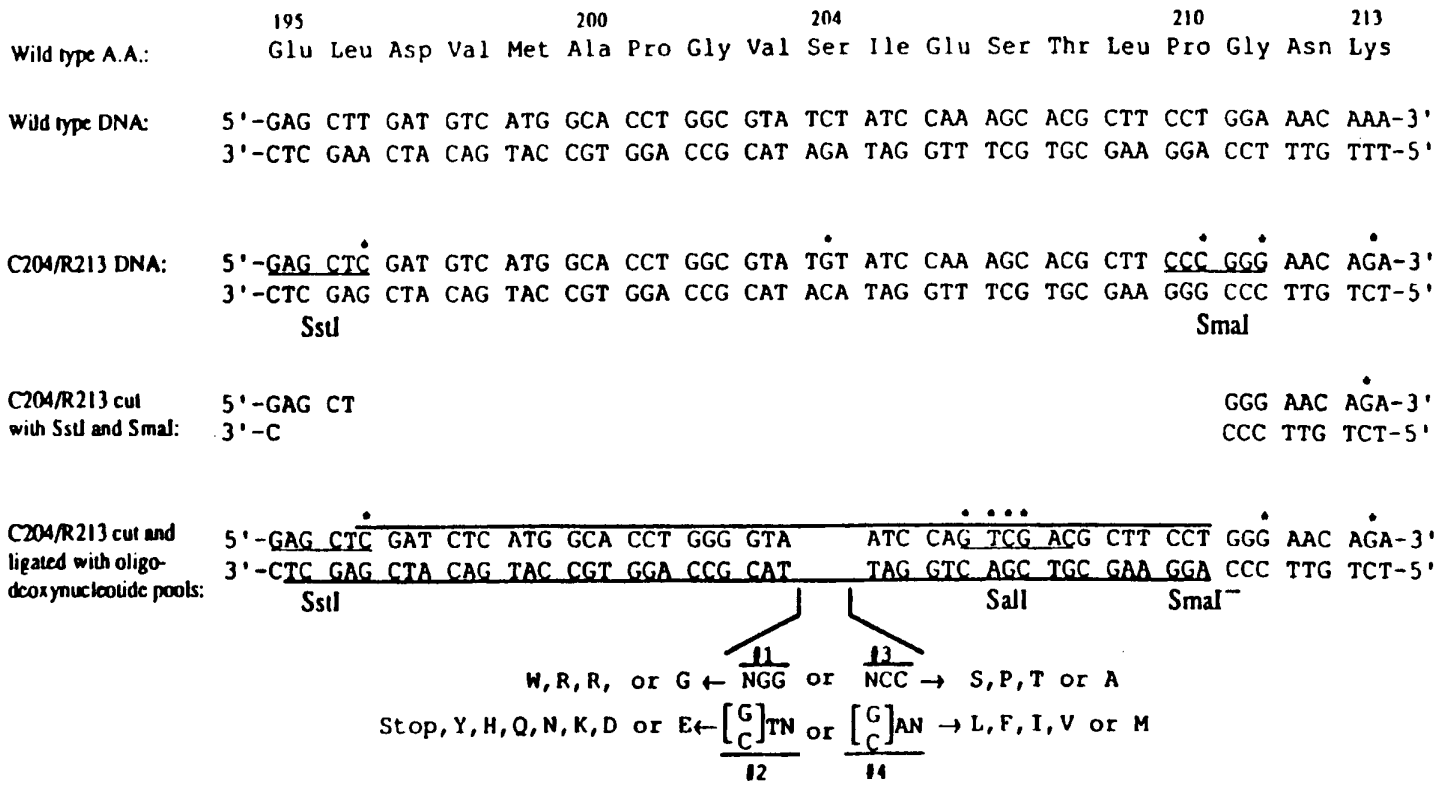

FIG.—37

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